EXPRESSION AND CHARACTERIZATION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS AND IGFBP-2 MRNA IN THE DEVELOPING CHICKEN EYE

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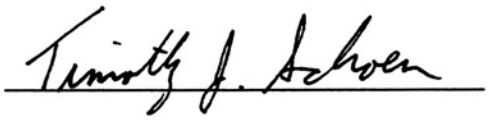

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ABSTRACT

Title of Dissertation: "Expression and Characterization of Insulin-Like Growth Factor Binding Proteins and IGFBP-2 mRNA in the Developing Chicken Eye"

Timothy James Schoen, Doctor of Philosophy, 1995

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The Insulin-Like Growth Factors (IGF-I and -II) are small, ~7 kDa, polypeptide hormones present in a variety of embryonic and adult tissues. Accumulating evidence indicates that they play a fundamental role in regulating embryonic growth and differentiation. IGFs are normally found in a complex with specific IGF-Binding Proteins (IGFBPs) of which six different types have been identified. Classically, IGFBPs have been shown to function as carriers of IGF, extending the half life of IGF in the circulation. However, recent evidence indicates that IGFBPs are able to either enhance or inhibit IGF effects on a number of different cell types, depending on the IGFBP. Ocular tissues have been shown to contain all of the components of the IGF system, including several different IGFBPs.

Chapter I provides a brief overview of ocular development and introduces the IGF system which consists of ligand,

receptor and binding protein. Chapter II provides evidence for an independent local regulation of IGFBPs in the vitreous humor of the developing chicken eye compared to the systemic regulation of circulating IGFBPs. The evidence supporting an independent regulation of vitreal IGFBPs includes: 1) a decrease in the level of vitreal IGF binding activity with increasing developmental age, whereas serum IGF binding shows an opposite pattern 2) a different developmental expression of vitreal IGFBPs as compared to serum IGFBPs, as assayed by western ligand blotting and 3) a unique glycosylation pattern of vitreal IGFBPs as compared to serum IGFBPs.

Chapter III describes the cloning of an avian cDNA and gene for IGFBP-2, the major IGFBP in ocular fluids and tissues. The deduced amino acid sequence of the avian IGFBP-2 cDNA along with the gene structure, including 5′ flanking sequence, is compared with IGFBP-2 sequences from several mammals. Chapter IV describes the results of northern blot and in-situ hybridization studies of IGFBP-2 expression in the developing ocular tissues of the chick. Finally, Chapter V presents a summary of the thesis findings and discusses potential future directions.

"EXPRESSION AND CHARACTERIZATION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS AND IGFBP-2 MRNA IN THE DEVELOPING CHICKEN EYE"

By

Timothy James Schoen

Dissertation submitted to the Faculty of the Department of Anatomy and Cell Biology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1995

DEDICATION

To my wife, Diane and children: Ryan, Heather, Jeremy and Travis, for their patience and support during my seemingly eternal career as a graduate student.

To my parents, Anne and Jim, who provided me with the fundamentals necessary to begin my quest for knowledge.

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CHAPTER I

Introduction

General Review of the Insulin-like Growth Factor System and its Potential Role in Ocular Development

Insulin-like growth factors

The insulin-like growth factors (IGFs) are a group of polypeptide hormones that have potent metabolic, proliferative and differentiative effects on a number of different tissues and cell types. In 1957, Salmon and Daughaday ascertained that in order for growth hormone to stimulate sulfate incorporation into cartilage, the presence of a serum derived factor was required, and was therefore named "sulfation factor activity" (SFA). A few years later, independent RIA studies revealed that there is much greater insulin activity present in serum than could be accounted for by the content of immunoreactive insulin, leading to the suggestion of a "non-suppressible insulin-like activity" (NSILA) (Froesch *et al.*, 1963). In the 1970's, it was discovered that cultured cells synthesized a "multiplication stimulation factor" (MSA) that is similar to insulin in activity (Dulak and Temins, 1973). All three of these activities (SFA, NSILA and MSA) were able to facilitate the effects of growth hormone and were therefore given the name somatomedins (Daughaday *et al.*, 1972).

Since their discovery, the somatomedins have been further categorized into two major classes: IGF-I and IGF-II

(Rinderknecht and Humbel, 1978a, 1978b). SFA and NSILA are identical and represent IGF-I, while MSA is now known to be IGF-II. Both IGF-I and IGF-II have molecular weights around 7500 kDa and are approximately 60% homologous to each other. In addition, both have regions homologous to the A and B chain of insulin, as well as to the C-peptide, present only in pro-insulin and an additional D chain (See Fig. 1) (Daughaday and Rotwein, 1989).

Accumulating evidence indicates that the IGF system plays an important role in the regulation of embryonic growth and differentiation. All of the components of the IGF system are present early in development of both mammals and birds. In mammals, where the embryo develops in close association with maternal tissues, IGF-I and IGF-II are present in both follicular (Rasasharma *et al.*, 1986) and oviduct fluids (Wiseman *et al.*, 1992). In addition, IGF is also highly expressed in decidual tissue, with the highest expression in the stroma (Croze *et al.*, 1990). In the non-placental chicken embryo, maternal IGF is stored in the yolk (Scavo *et al.*, 1989).

Both IGF-I and IGF-II or their mRNAs are present in embryonic tissues of the: chicken (Serrano *et al.*, 1990; Kikuchi *et al.*, 1991), mouse (D'Ercole *et al.*, 1980), rat (Rotwein *et al.*, 1987) and human (Sara *et al.*, 1981; Han *et al.*, 1988) as well as several other species. The expression of IGF appears to be both spatially and temporally regulated. In the chicken embryo, IGF-I mRNA is differentially expressed in several embryonic tissues including the eye and brain

(Kikuchi *et al.*, 1990). In this same study, IGF mRNA was undetectable in embryonic liver suggesting that extrahepatic IGF synthesis may account for most of the IGF present in the embryo. *In situ* hybridization studies of embryonic rat tissue indicate a spatial separation of IGF-I and IGF-II mRNA, with IGF-I mRNA being concentrated in regions of mesenchyme surrounding developing muscle and cartilage, while IGF-II mRNA is more abundant in mesodermal tissue such as muscle, cartilage and blood vessels (Bondy *et al.*, 1990). Besides the spatial separation of IGF-I and -II mRNA there are also temporal differences in the appearance of the peptide in the serum during development. In most mammals, IGF-I serum levels are low in the fetus and increase substantially following birth (Daughaday and Rotwein, 1989). Likewise, IGF-I is found as early as embryonic day 6 in chicken serum (Robcis *et al.*, 1991). Here, IGF levels increase markedly from day 8 to day 15, increasing again after hatching.

Receptors for the insulin-like growth factors

The biological actions of both IGF-I and IGF-II are mediated through specific receptors. The type I (IGF-I) receptor is responsible for mediating most of the actions of both IGF-I and IGF-II. The type I IGF receptor is similar to the insulin receptor in that it contains two extracellular α subunits (130 kDa) responsible for binding ligand and two intracellular ß subunits (90 kDa) containing a tyrosine kinase domain responsible for the phosphorylation of intracellular proteins (Ulrich *et al.*, 1986). Hybrid receptors containing

one insulin receptor alpha and beta subunit and one IGF-I receptor alpha and beta subunit have also been identified (Soos *et al.*, 1990). These receptors appear to have a higher affinity for IGF-I than insulin, however the overall distribution of hybrid receptors in various tissues remains to be determined.

The type 2 (IGF-II) receptor is identical to the mannose-6-phosphate receptor (250 kDa) that is involved in the targeting of lysosomal enzymes (Morgan *et al.*, 1987). IGF-II receptors have no intrinsic kinase activity, do not bind insulin and have a low affinity for IGF-I. The role of the IGF-II receptor remains to be determined since it is thought that the majority of the biological actions of IGF-I and IGF-II are mediated through the IGF-I receptor (Czech MP, 1989). A schematic diagram of the structure of the insulin, IGF-I and IGF-II receptors is shown in Fig. 2.

Although the precise mechanism by which IGF is able to induce metabolic, mitogenic and differentiative effects is unknown, the binding of IGF to the IGF-I receptor is associated with the phosphorylation of endogenous cellular substrates.

The binding of ligand to the IGF-I receptors extracellular alpha subunits results in a conformational change in the cytoplasmic beta subunits which results in the autophosphorylation of beta subunit tyrosine residues. The autophosphorylation of the beta subunits produces an enhanced tyrosine kinase activity towards other cellular substrates. One of the best characterized substrates is known as the

Insulin Receptor Substrate-1 (IRS-1) (Sun *et al.*, 1991). IRS-1 is involved in interactions with other cellular proteins through src-homology 2 (SH2) domains (Cantley, L.C. *et al.*, 1991). However, further investigation is needed to clarify the link between substrate phosphorylation and the resulting metabolic, mitogenic and differentiative effects of IGF.

In situ binding studies have demonstrated that IGF-I receptors are present as early as gastrulation in the developing chicken (Girbau *et al.*, 1989). During neuralation, these receptors appear to be widely dispersed throughout the immature nervous system. Furthermore, they appear to be functional as both the ß-subunit of the IGF-I receptor as well as exogenous substrate are phosphorylated in the presence of IGF (Girbau *et al.*, 1989). In the mouse embryo, IGF-I and IGF-II receptors are detectable as early as the blastula stage using radioactive ligand (Mattson *et al.*, 1988). Using the more sensitive RT-PCR technique, both IGF-I and insulin receptor transcripts are detected as early as the eight cell stage (Rappolee *et al.*, 1990). Although data are lacking regarding IGF receptors in the early developing human fetus, IGF-I receptors have been identified in brain, liver, kidney, lung and adrenals of 10-17 week old fetuses (Sara *et al.*, 1983).

Insulin-like growth factor binding proteins

The initial purification of the IGFs led to the discovery that they were carried by specific high affinity IGF binding proteins (IGFBPs). IGFBPs are a heterogeneous

population of proteins, with molecular weights ranging from 25 kDa to 150 kDa. At present, six types of IGFBPs have been identified based on their nucleotide and amino acid sequence (Shimasaki *et al.*, 1991a; Shimasaki *et al.*, 1991b). All of the IGFBPs are soluble proteins that are secreted, making it difficult to assess their intracellular levels. All have a high affinity for IGF-I and IGF-II, with virtually no insulin binding activity. In addition, all IGFBPs contain 18 cysteines that are aligned in the amino and carboxyl-termini. These termini exhibit the highest degree of sequence conservation between IGFBPs. In contrast, the cysteine-free central regions show the least conservation. An alignment of the most conserved regions of the 6 human IGFBP sequences is shown in Fig. 3.

The first IGFBP to be identified was IGFBP-1. This was first discovered in amniotic fluid (Chochinov *et al.*, 1977) but later found to be present in fetal serum (Povoa *et al.*, 1984) and in the conditioned medium from Hep G2 hepatoma cells (Lee *et al.*, 1988). IGFBP-1 has a molecular weight of approximately 28-31 kDa and although there are no N-linked glycosylation sites, several potential O-linked glycosylation sites are present which may account for the 4.3% carbohydrate content (Bohn and Kraus, 1980). The gene for IGFBP-1 has been identified and localized to human chromosome 7 (Ekstrand *et al.*, 1990). Northern hybridization studies indicate that mRNA for IGFBP-1 is restricted to placental tissue and fetal liver (Brinkman *et al.*, 1988). An interesting feature of the IGFBP-1 molecule is that it contains the Arg-Gly-Asp (RGD) peptide

sequence that has been shown to bind to cell surface integrins (Hynes, 1987). The protein also contains a region rich in proline, glutamate, serine and threonine (PEST). The PEST sequence has been found in several other proteins that exhibit a rapid turnover such as c-myc and c-fos and which may regulate susceptibility to proteolytic degradation (Rogers *et al.*, 1986).

IGFBP-2 was first characterized in conditioned medium from Buffalo rat liver (BRL) cells (Mottola *et al.*, 1986). The protein has a molecular weight around 31 kDa, is non-glycosylated and, like IGFBP-1, contains the RGD peptide, but not the PEST sequence. The human gene for IGFBP-2 has been identified (Binkert *et al.*, 1989) and localized to chromosome 2 (Agarwal *et al.*, 1991). In addition to containing the RGD sequence, IGFBP-2 exhibits a Zn^{+2} finger motif and a putative ATP binding site that may be involved in the regulation of ligand binding (Agarwal *et al.*, 1991). Of the six IGFBPs, IGFBP-2 exhibits the highest degree of conservation between the various mammalian species (Delhanty and Han, 1992). IGFBP-2 is present in a variety of different tissues and fluids and is expressed at high levels during development (Ooi *et al.* 1990; Orlowski *et al.* 1990). In ocular tissues and fluids, IGFBP-2 appears to be the predominant IGFBP (Waldbillig *et al.*, 1991; Arnold *et al.*, 1992) and is probably synthesized locally since ocular tissues contain abundant IGFBP-2 mRNA (Agarwal *et al.*, 1991, Arnold *et al.*, 1993). In addition, vitreal IGFBP-2 levels exhibit an independent regulation from its serum counterpart during development

(Schoen *et al.*, 1992; Yang *et al.*, 1993) as well as under pathological conditions (Waldbillig *et al.*, 1994).

IGFBP-3 is the major carrier of IGF in the circulation (Baxter and Martin, 1989). In its native state, it exists as a 150 kDa complex that breaks down under acidic conditions to yield an acid stable BP of 50-60 kDa and an acid labile subunit of around 80 kDa (Baxter and Martin, 1987). IGFBP-3 does not contain the RGD nor PEST sequence, but does appear to be N-glycosylated (Baxter *et al.*, 1986). Northern blot analysis of adult tissues shows that IGFBP-3 mRNA is highly expressed in the liver (Shimasaki *et al.*, 1989), but is also expressed at a high level in a number of other tissues including the cornea (Arnold *et al.*, 1992). The gene for IGFBP-3 is located on human chromosome 7, only 20 kb from the gene for IGFBP-1 (Shimasaki *et al.*, 1991a).

IGFBP-4 is a 25-30 kDa IGFBP, first isolated from the conditioned medium of a human osteosarcoma cell line (Mohan *et al.*, 1989). Sequence analysis indicates that IGFBP-4 has two cysteines in the mid-region of the molecule, in addition to the conserved 18 cysteines. It exhibits only 35% identity to IGFBP-1, -2, or -3 (LaTour *et al.*, 1990). A single N-linked glycosylation site is present and the reported variations in the size of the protein appear to be due to differential glycosylation of this site (Cheung *et al.*, 1991). The gene for IGFBP-4 has been mapped to human chromosome 17 (Shimasaki, et al, 1991a) and a single mRNA transcript of 2.6 kb is present in multiple tissues of the rat, with the liver exhibiting the highest expression (Shimasaki, et al, 1990).

The IGFBP-5 protein was first isolated from rat serum and the cDNA from a rat ovary and human placental library (Shimasaki *et al.*, 1991a). The protein has a molecular weight of 29 kDa, with no N-linked glycosylation sites. The human form of IGFBP-5, which varies from the rat in 5 of the first 15 amino acids in the N-terminal region, was copurified with a protease that cleaves the protein at its C-terminal to yield a smaller 24 kDa product (Bautista *et al.*, 1991). Northern blot analysis of rat tissues showed IGFBP-5 is present in brain, heart, liver, spleen, adrenal, intestine, lung, testis and kidney (Shimasaki *et al.*, 1991a). The gene for IGFBP-5 has been mapped to human chromosome 5.

IGFBP-6 was first isolated from cerebrospinal fluid in 1989 (Roghani *et al.*, 1989). However, the complete cDNA sequence obtained from a rat ovary and human placental library was not reported until 1991 (Shimasaki *et al.*, 1991c). IGFBP-6 has a molecular weight of approximately 34 kDa and does not appear to be glycosylated. Northern blot analysis of rat tissues reveals a single transcript of 1.3 kb in brain, liver, lung, heart, lung, spleen, kidney, stomach intestine and testis, with the highest expression in lung (Shimasaki *et al.*, 1991c). The gene for IGFBP-6 has been mapped to human chromosome 12 (Shimasaki *et al.*, 1991b).

An explanation for having six similar but different IGFBPs is not clear. However, it appears that like other abundant proteins, the IGFBPs contain multiple genes that most likely arose through duplication of a single ancestral gene. The presence of multiple copies of a gene has several

advantages. First of all, having multiple copies of a gene relaxes the evolutionary pressure on its sequence. This allows mutation of one of the genes without affecting the expression of the normal copy. In addition, if the mutation is beneficial, it will most likely be selected for by evolution. The finding that some IGFBPs appear to enhance the actions of IGF, while others inhibit it, demonstrate the unique properties of each of the individual IGFBPs and may be the result of beneficial mutations.

Although IGFBPs are present in just about all tissue types, certain IGFBPs predominate over others in a particular tissue type. For example, IGFBP-1 is the predominant IGFBP in female reproductive tissues, whereas, IGFBP-3 appears to be the major IGFBP produced by the liver. This tissue specificity may be regulated by interactions of tissue-specific sequences in the 5' upstream sequence with tissue-specific nuclear transcription factors. In summary, the presence of multiple IGFBP genes that code for proteins that are differentially expressed in tissues and exhibit differences in affecting the activity of IGF, reinforces the idea that IGFBPs are functionally important proteins.

Proteolysis as a means of regulating the activity of insulin-like growth factor binding proteins.

Besides regulating the expression of IGFs and their receptors and binding proteins, another means of modulating IGF activity appears to be the selective destruction of IGFBPs by specific proteases. The first such IGFBP protease to be

identified was an IGFBP-3 protease in the serum of pregnant women (Hossenlopp *et al.*, 1990; Guidice *et al.*, 1990). However, other studies have shown that IGFBP proteolytic activity is systemically elevated in a number of physiologically stressful conditions such as surgery (Hughes *et al.*, 1992; Davenport *et al.*, 1992), severe illness (Davies *et al.*, 1991) and chronic renal failure (Lee *et al.*, 1993). In addition, other IGFBP-specific proteases such as an IGFBP-4- specific protease produced by dermal fibroblasts have been identified (Fowlkes and Freemark, 1992). Similarly, cultured rat granulosa cells synthesize a protease that acts on a 28-29 kDa IGFBP presumed to be IGFBP-5 (Fielder *et al.*, 1993).

An IGFBP-3-specific protease has also been identified in the vitreous humor (Schoen *et al.* 1995). The IGFBP-3ase activity is inhibited by heat or cold, has a pH optimum of about 8.0 and is inhibited by EDTA. Incubation of recombinant IGFBP-3 or serum with partially-purified IGFBP-3ase results in the appearance of low molecular weight fragments of approximately 30 kDa. These fragments are undetectable by western ligand blotting but are readily visualized using an IGFBP-3-specific antibody. An additional interesting finding is that there is an increased amount of IGFBP-3 proteolytic fragments in the diabetic vitreous humor as compared to controls (Waldbillig *et al.* 1994).

Although the precise function of the IGFBP-specific proteases is not known, their ability to specifically degrade and/or lower the affinity of IGFBPs for IGF could result in a

greater "free" concentration of IGFs at target tissue sites, thus facilitating IGF actions. Alternatively, since some IGFBPs facilitate the action of IGF (see next section), their degradation by proteases might result in decreased rather than increased IGF activity. Clearly, more work is needed to elucidate the role of IGFBP-specific proteases in the regulation of IGF activity.

Functional significance of insulin-like growth factor binding proteins

Most IGF-I and IGF-II is transported in the circulation by IGFBP-3. Since IGF is not stored in secretory granules but, rather, is released into the circulation upon synthesis, it has been hypothesized that the IGFBPs act to protect IGF from being excreted and also serve as a reservoir for IGF activity (Guler *et al.*, 1987). In fact, the half-life of IGF-I in the circulation is extended approximately 10-fold by binding to IGFBPs (Guler *et al.*, 1987). An additional feature to consider is the finding that the total concentration of both IGF-I and IGF-II in the serum of adult humans is approximately 1000 fold higher than the insulin concentration (Baxter *et al.*, 1986). Because of IGFs ability to cross-react with insulin receptors, the free circulating IGF should produce a severe hypoglycemia. However, since >95% of all IGF in the circulation is bound to IGFBP-3, the amount of free IGF available for cross-reacting with insulin receptors is negligible.

Recent evidence indicates that, besides the above

mentioned roles, IGFBPs are capable of directly modulating the activity of IGF. Depending on the cell type and the individual IGFBP, the activity of IGF can either be enhanced or inhibited. For example, IGFBP-1 is both a positive and negative regulator of IGF action; it facilitates the action of IGF-I in stimulating DNA synthesis in aortic smooth muscle cells (Elgin *et al.*, 1987) but inhibits IGF-I mediated DNA synthesis in choriocarcinoma cells (Ritvos *et al.*, 1988). To date, IGFBP-2 has been found to be both stimulatory, as well as inhibitory. In both carcinoma cells and smooth muscle cells, IGFBP-2 has been shown to facilitate the effect of IGF-I on DNA synthesis (Bourner, *et al*, 1992; Chen *et al.*, 1994). In contrast, IGFBP-2 inhibits the binding of radiolabeled IGF-I and -II to small cell lung carcinoma cells and inhibits IGF-I stimulated DNA synthesis by these cells as well (Reeve *et al.*, 1993). IGFBP-3 has also been reported to act as either a positive or negative modulator of IGF activity. When IGFBP-3 is added with IGF-I to cultured fibroblasts, a decrease in DNA synthesis is observed. However when added first and allowed to adhere to cell surfaces, there is a 35% potentiation of IGF-induced DNA synthesis (DeMellow and Baxter, 1988). IGFBP-4 appears to inhibit the actions of IGF (Mohan *et al.*, 1989), while IGFBP-5 and -6 acts to enhance IGF-I stimulated mitogenesis (Andress and Birnbaum, 1991).

Besides influencing the activity of IGF, it appears that IGFBPs may exhibit activity independent of IGF. An IGFBP (presumably IGFBP-2) isolated from vascular endothelial cells is capable of stimulating aminoisobutyric acid (AIB) uptake by

cultured endothelial cells in the absence of IGF-I (Booth *et al.*, 1990). The RGD peptide which is present in IGFBP-1 and -2 and which enables these proteins to "dock" to cell surface integrins may be one means for facilitating IGF actions. In support of this idea, recombinant IGFBP-1 is able to stimulate the migration of Chinese hamster ovary (CHO) cells in the presence or absence of IGF-I (Jones *et al.*, 1993). Affinity purification of CHO membrane proteins using IGFBP-1 identified α_5 β_1 integrin as the only cell surface molecule capable of binding IGFBP-1. In addition, mutation of the RGD to WGD resulted in the complete loss of its activity, indicating that the effect of IGFBP-1 on cell migration is actually mediated through α_5 β_1 integrin.

IGF binding proteins in embryonic development.

Using RT-PCR, IGFBP mRNAs have been detected in mammalian embryos as early as pre-implantation (Schultz *et al.*, 1993). In these studies, mRNA for IGFBP-2 -3, and -4 were detected in the mouse from the oocyte stage through the blastocyst stage. In contrast, IGFBP-5 and -6 were detected in the ovary but not in the oocyte or pre-implantation embryo until the blastocyst stage.

At later stages of development, IGFBPs are present in a variety of different tissues. Northern blot studies of mRNA levels in the developing rat show the liver has the highest expression of IGFBP-1, which appears to peak just after birth and then decreases 10 fold by adulthood (Ooi *et al.*, 1990). *In situ* hybridization studies of the developing rat embryo

show that IGFBP-2 exhibits a temporal and spatial developmental expression pattern distinct from that of IGF-I and -II (Wood *et al.*, 1990; Lee *et al.*, 1992). This is most apparent in the developing limb bud, where IGFBP-2 mRNA is localized to the apical ectodermal ridge (AER) and IGF-I and -II mRNA transcripts are found in the adjacent mesoderm (Streck *et al.*, 1992). Similarly, a distinct localization of IGFBP-5 and IGF-I has been demonstrated in embryonic rat brain (Bondy and Lee, 1993). In the embryonic rat, *in situ* hybridization studies reveal that IGFBP-3 mRNA expression is high in the urogenital tract, several muscle groups and the nasal epithelium (Cerro *et al.*, 1993). This same study showed IGFBP-4 to be expressed in many tissues with the exception of the spinal cord, specific cartilage groups and the thymic cortex. In parallel, IGFBP-6 is found to be expressed by embryonic day 14 in the liver and a previously unrecognized cells surrounding cartilage.

Ocular development

Eye development begins with the formation of the optic vesicles which originate as outpouchings of the lateral wall of the diencephalon (Fig. 4, A, B). The optic vesicles grow laterally toward the surface ectoderm while the proximal end of the vesicle constricts to form the optic stalk (presumptive optic nerve). Upon contact with the surface ectoderm, the vesicle invaginates to create a double-layered optic cup, with the outer layer forming the retinal pigmented epithelium (RPE) and the inner layer forming the neural retina (Fig. 4C). The

neural retina continues to differentiate into specific layers containing the ganglion, bipolar, amacrine, horizontal, Muller and photoreceptor cells. The choroid is situated beneath the pigmented epithelium and carries the main blood supply for the retina. It develops from the condensation of neural crest cells that surround the optic cup.

Like the choroid, the sclera develops predominantly from neural crest cells that surround the optic cup. These cells differentiate into fibroblast-like cells that synthesize abundant collagen fibrils. The sclera is penetrated by nerves and blood vessels, and in the chicken, hyaline cartilage forms at certain positions between the choroid and sclera.

The vitreous humor consists of a clear gelatinous fluid that fills the central cavity of the eye and stabilizes the retina and other structures from sudden movements. The primary vitreous humor consists of fibrillar material, mesenchymal cells and the hyaloid vascular system, that, in mammals, serves as a temporary blood supply to lens. Later in development, the primary vitreous humor is replaced by secondary vitreous humor. Secondary vitreous humor is secreted by the retina, ciliary body and also fibroblast-like cells within the vitreal cavity known as hyalocytes. The secondary vitreous humor consists mostly of hyaluronic acid and collagen fibrils.

The anterior-most edges of the optic cup differentiate into parts of the ciliary body and iris. The ciliary body forms from neuroectoderm (inner and outer layers of the optic

cup), neural crest and mesodermal components. The ciliary body is a secretory structure that is involved in the production of aqueous humor. In addition, it regulates accommodation of the lens. Contraction of the ciliary muscle results in a loosening of zonule fibers that extend from the edge of the ciliary body to the perimeter of the lens. The increased slack in the zonule fibers allows the lens to change from an elliptical to a more round shape that has a greater index of refraction.

The iris is derived from neuroectoderm, neural crest cells and mesodermal tissue. Initially, neural crest cells migrate from the rim of the optic cup between the corneal endothelium and the lens epithelium, forming a pupillary membrane. The two layers of neuroectoderm at the anterior rim of the optic cup then migrate across the pupillary membrane and fuse with it to form the iris. Mesodermally- derived vascular components then invade the region between the two layers. Both the sphincter and dilator muscles are thought to originate from neuroectodermal tissue.

Upon contact with the optic cup, the surface ectoderm thickens to form the lens placode. This gives rise to lens vesicle, which invaginates into the optic cup and loses its attachment to the surface ectoderm. The posterior cells of the lens vesicle elongate, stop dividing, lose their nuclei and begin to produce crystallins. The anterior cells of the lens retain their ability to divide and produce a thick basal lamina known as the lens capsule. Initially, the lens receives nourishment from a specialized vascular system known

as the *tunica vasculosa lentis*, which regresses at latter stages of development.

The region of the surface ectoderm from which the lens vesicle detached consists of a superficial epithelial layer and a basal cuboidal epithelial layer. The basal cell layer, which becomes the corneal epithelium, secretes the primary stroma consisting of collagen fibrils and glycosaminoglycans. Following the formation of the primary stroma, neural crest cells migrate in and differentiate into the endothelium and keratocytes of the corneal stroma.

The insulin-like growth factor system in ocular development.

The development and differentiation of ocular tissues is a complex process that is dependent upon the interaction of multiple cell types and the expression of numerous genes. The finding that components of the IGF system are present in several ocular tissues during development, strongly suggests a role for the IGF system in ocular development.

IGF-I has been identified in several ocular tissues (Hansson *et al.*, 1989; Danias and Stylianopoulou, 1990;) and is also present in the vitreous humor of normal and diabetic individuals (Grant *et al.*, 1986). In addition, an IGF-I-like protein that induces lens differentiation *in-vitro*, has been identified in embryonic vitreous humor (Beebe *et al.*, 1980; Beebe *et al.*, 1987).

IGF-I receptors are present in a number of different developing ocular tissues of the chicken, including the lens, sclera, retina and retinal pigmented epithelium (Bassas *et*

al., 1987; Zick *et al.*, 1987; Ocrant *et al.*, 1989; Waldbillig *et al.* 1990 and Bassnett and Beebe, 1990). Depending on the specific ocular tissue, IGF-I receptors exhibit a differential developmental expression. In the sclera, for example, the number of IGF-I receptors decreases by approximately 50% between embryonic day 10 and the 2nd week after hatching whereas a 50% increase is observed for pigment epithelium IGF-I receptors (Waldbillig *et al.*, 1991).

Several different IGFBPs have been identified in adult vitreous humor (Ocrant *et al.*, 1989; Arnold *et al.*, 1993), although virtually nothing has been reported in the scientific literature regarding their developmental regulation. Because of their known importance in regulating the actions of the IGFs and an increasing number of studies reporting intrinsic biological activity of IGFBPs, I decided to examine these proteins during ocular development.

The first part of my thesis project involved examining the similarities and differences in the developmental regulation, structure and glycosylation of vitreal and serum IGFBPs. In order to examine the developmental expression of IGFBP-2 in ocular tissues, it was necessary to obtain the chicken IGFBP-2 cDNA sequence. Therefore, the second part of the project dealt with acquiring the chicken cDNA. Finally, the third part of the dissertation work involved examining the developmental expression of IGFBP-2 in ocular tissues by both northern blotting and *in situ* hybridization.

Fig. 1 **Schematic structure of pro-insulin, insulin-like growth factor and insulin.** (from Adamo *et al.*, 1992).
The insulin-like growth factors (IGF-I and IGF-I) are closer in structure to pro insulin than to insulin, as both contain the C peptide. The D peptide is only present in the IGFs. The A and B chain of all three ligands are linked by disulfide (S-S) bonds.

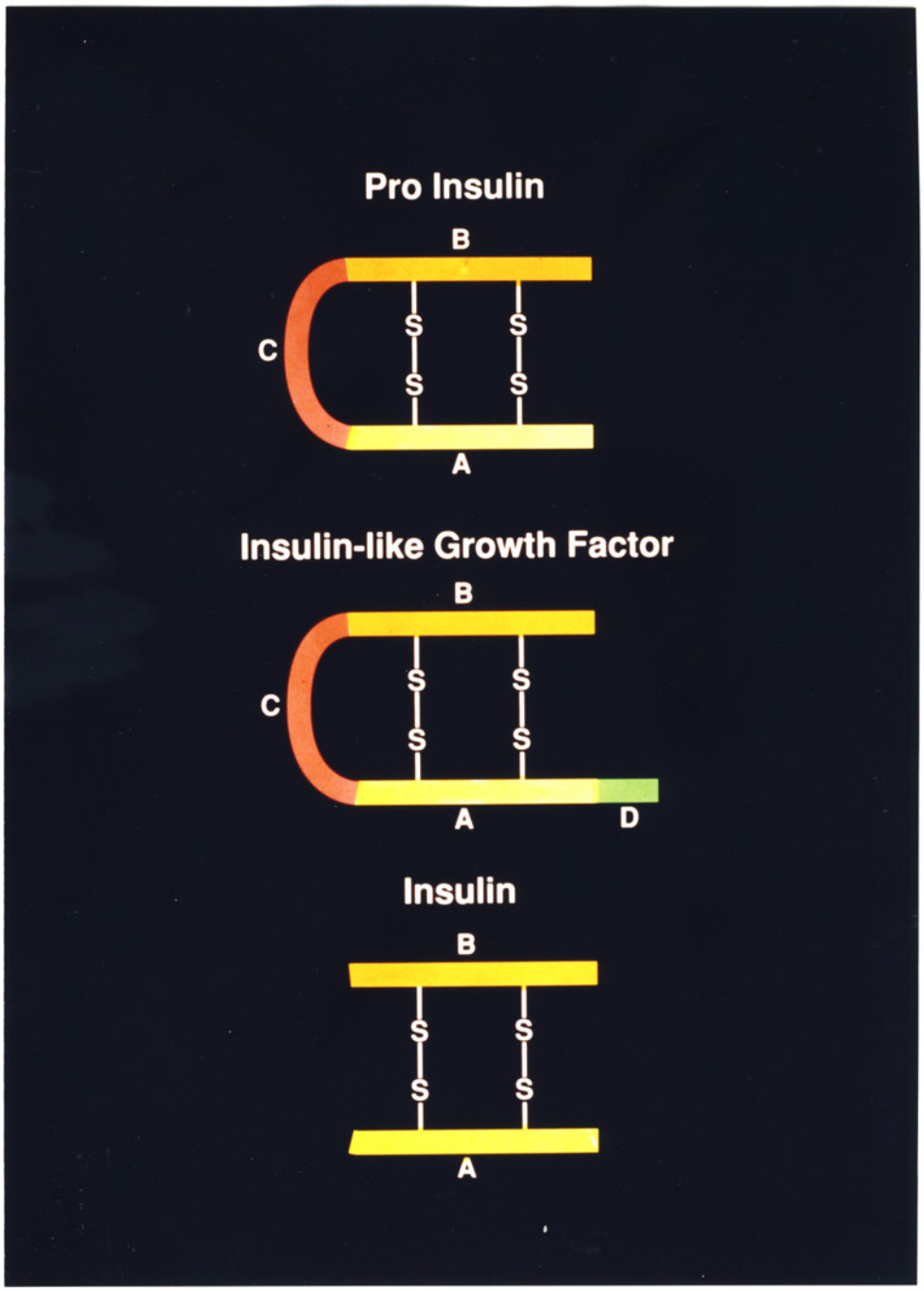

Pro Insulin
B
C
S
S
S
S
A
Insulin-like Growth Factor
B
C
S
S
S
S
A
D
Insulin
B
S
S
S
S
A

Fig. 2 **Schematic representation of the insulin, IGF-I (A) and IGF-II (B) receptors.**
(adapted from Heyner and Garside, 1994)

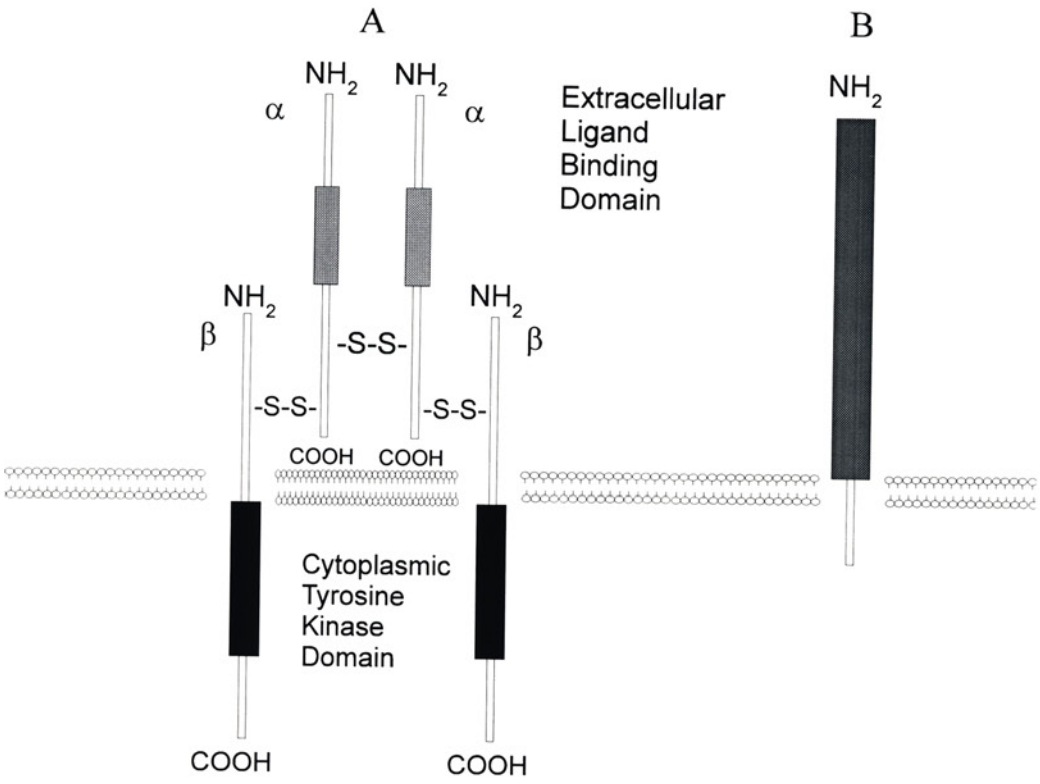

A
B
NH2
NH2
α
α
Extracellular
Ligand
Binding
Domain
NH2
NH2
β
β
-S-S-
-S-S-
-S-S-
COOH
COOH
Cytoplasmic
Tyrosine
Kinase
Domain
COOH
COOH

Fig. 3 **Amino acid alignment of the most conserved regions from the six human IGFBPs.** Conserved cysteine residues are shown in yellow.

(from Shimasaki and Ling, 1991c)

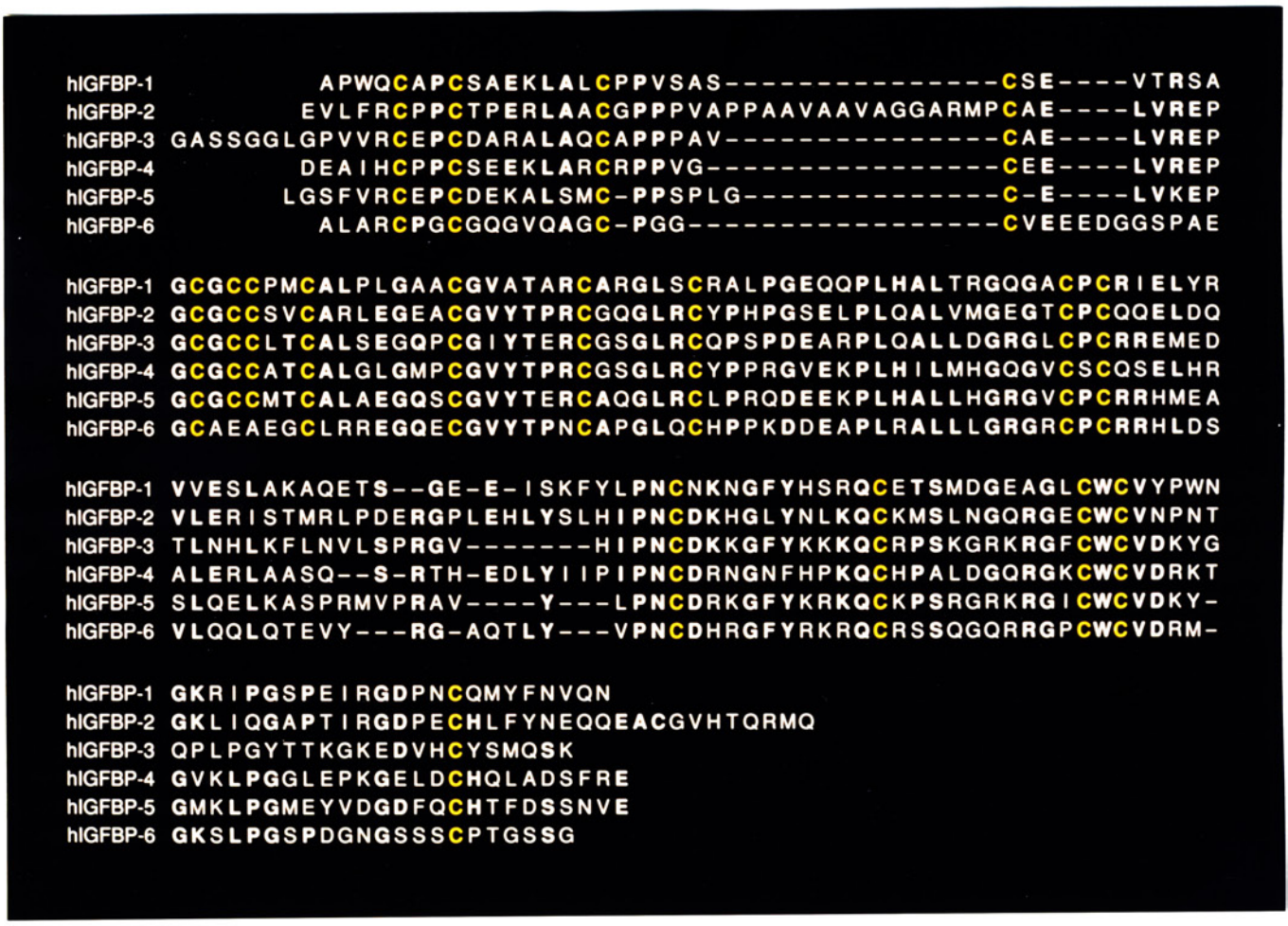
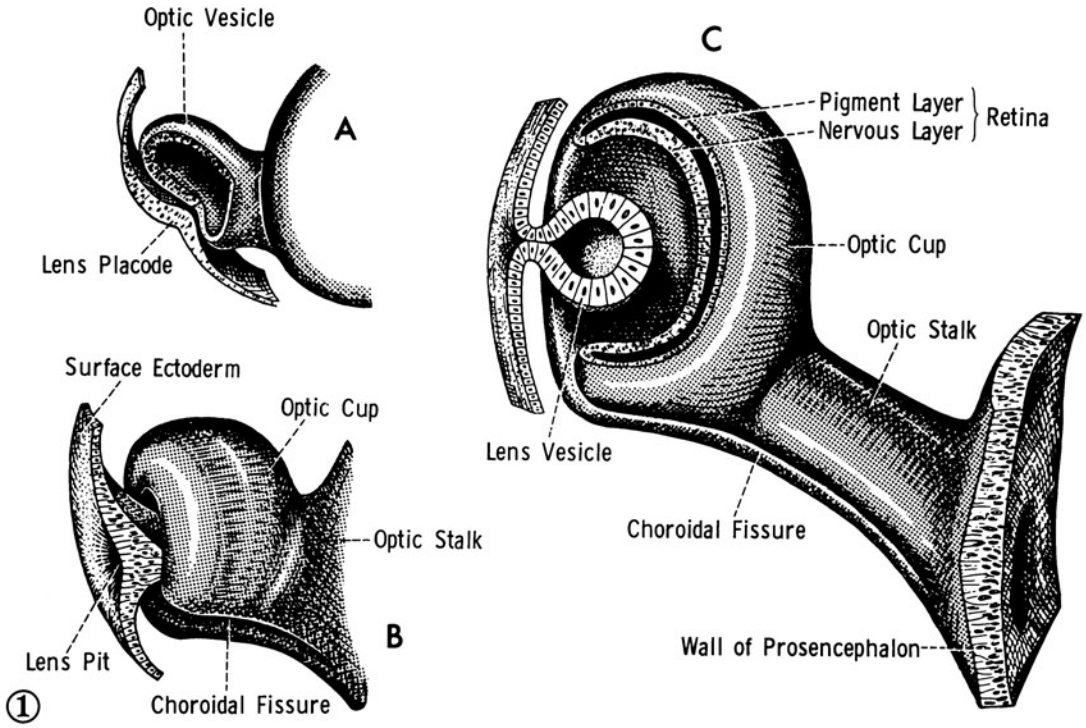

hIGFBP-1 APWQCAPCSAEKLALCPPVSAS----------CSE----VTRSA
hIGFBP-2 EVLFRCPPCTPERLAACGPPPVAPPAAVAAVAGGARMPCAE----LVREP
hIGFBP-3 GASSGGLGPVVRCEPCDARALAQCAPPPAV----------CAE----LVREP
hIGFBP-4 DEAIHCPPCSEEKLARCRPPVG----------CEE----LVREP
hIGFBP-5 LGSFVRCEPCDEKALSMC-PPSPLG----------C-E----LVKEP
hIGFBP-6 ALARCPGCGQGVQAGC-PGG----------CVEEEDGGSPAE
hIGFBP-1 GCGCCPMCALPLGAACGVATARCARGLSCRALPGEQQPLHALTRGQGACPCRIELYR
hIGFBP-2 GCGCCSVCARLEGEACGVYTPRCGQGLRCYPHPGSELPLQALVMGEGTCPCQQELDQ
hIGFBP-3 GCGCCLTCALSEGQPCGIYTERCGSGLRCQPSPDEARPLQALLDGRGLCPCRREMED
hIGFBP-4 GCGCCATCALGLGMPCGVYTPRCGSGLRCYPPRGVEKPLHILMHGQGVCSCQSELHR
hIGFBP-5 GCGCCMTCALAEGQSCGVYTERCAQGLRCLPRQDEEKPLHALLHGRGVCPCRRHMEA
hIGFBP-6 GCAEAEGCLRREGQECGVYTPNCAPGLQCHPPKDDEAPLRALLLGRGRCPCRRHLDS
hIGFBP-1 VVESLAKAQETS--GE-E-ISKFYLPNCNKNGFYHSRQCETSMDGEAGLCWCVYPWN
hIGFBP-2 VLERISTMRLPDERGPLEHLYSLHIPNCDKHGLYNLKQCKMSLNGQRGECWCVNPNT
hIGFBP-3 TLNHLKFLNVLSPRGV-------HIPNCDKKGFYKKKQCRPSKGRKRGFCWCVDKYG
hIGFBP-4 ALERLAASQ--S-RTH-EDLYIIPIPNCDRNGNFHPKQCHPALDGQRGKCWCVDRKT
hIGFBP-5 SLQELKASPRMVPRAV----Y---LPNCDRKGFYKRKQCKPSRGRKRGICWCVDKY-
hIGFBP-6 VLQQLQTEVY---RG-AQTLY---VPNCDHRGFYRKRQCRSSQGQRRGPCWCVDRM-
hIGFBP-1 GKRIPGSPEIRGDPNCQMYFNVQN
hIGFBP-2 GKLIQGAPTIRGDPECHLFYNEQQEACGVHTQRMQ
hIGFBP-3 QPLPGYTTKGKEDVHCYSMQSK
hIGFBP-4 GVKLPGGLEPKGELDCHQLADSFRE
hIGFBP-5 GMKLPGMEYVDGDFQCHTFDSSNVE
hIGFBP-6 GKSLPGSPDGNGSSSCPTGSSG

Fig. 4 **Schematic diagram of eye formation.** A. The first stages of eye formation in which the optic vesicle outpouches from the diencephalon. B. Invagination of surface ectoderm into the optic cup. C. Formation of lens vesicle and demonstration of the layers of the retina.

(from Tripathi and Tripathi *et al.*, 1984)


Optic Vesicle
A
Lens Placode
Surface Ectoderm
Optic Cup
Optic Stalk
B
Lens Pit
Choroidal Fissure
①
C
Pigment Layer
Nervous Layer
Retina
Optic Cup
Optic Stalk
Lens Vesicle
Choroidal Fissure
Wall of Prosencephalon

CHAPTER II

Materials and Methods

Animals

Chicken eggs were purchased from Truslow Farms (Chestertown, Maryland) and staged according to Hamburger and Hamilton, 1951. The IGF binding assay, affinity crosslinking, western ligand blotting and deglycosylation experiments were performed on vitreous humor and serum pooled from a number of animals which varied with the developmental stage. 60 chicken embryos were pooled each for E-6, E-12 and E-19, respectively and 12 chickens each were pooled for P-2 and P-7. For animals that were 2 to 12 weeks old, 4 chickens were used for each age. A minimum of two different pools of samples were utilized in each of the binding assays, crosslinking and western ligand blot experiments, i.e. at least two independent experiments were performed to establish each parameter.

For the quantitative northern blot experiments, three separate batches of eggs were used to avoid possible problems from individual batch variations. A total of 60 embryos were used for E-8, 40 for E-12, 30 for E-18 and 20 for post-hatching day 2.

Vitreous humor and Serum Preparation

Embryonic chickens were decapitated and the heads rinsed with phosphate-buffered saline (PBS; pH 7.4) to remove

any contaminating blood. The eyes were enucleated and the anterior portion (cornea, lens, iris) of the eye removed. The vitreous humor body was removed from the posterior eye cup using fine forceps, stored on ice during collection and pooled. Pooled vitreous humor was centrifuged (4°C; 12,000 x g; 15 min) and the supernatant (vitreous humor) was stored at -20°C. Because the vitreous humor body of older birds (e.g. 3-12 week) contained a distinctive gel phase component, the vitreous humor bodies were sonicated (Ultrasonics model W225 sonicator, Farmington, NY) for 15 seconds prior to centrifugation.

Serum was collected from embryos by inserting a pulled glass capillary pipette into extra-embryonic blood vessels or directly into the heart. Adult animals were sacrificed by decapitation and exsanguinated. The blood was allowed to clot at room temperature for one hour before centrifuging at 12,000 x g for 15 min, after which the serum was collected and stored at -20°C.

IGF binding assay

Fifty μl of sample was incubated with 100 μl of ^{125}I-IGF-II or ^{125}I-IGF-I (Specific Activity= 2000 Ci/mmol)(Amersham, Arlington Heights, Il) and 50 μl of unlabeled IGF-I, or unlabeled IGF-II (70 pM) (Amgen Biologicals, Thousand Oaks, CA) or calcium free Krebs Ringer Phosphate (KRP) buffer containing 0.1% BSA (pH 8.0). Stock radiolabeled IGF (IGF-I and II) was in KRP containing 3.0% bovine serum albumin (BSA) and 3.0 mg/ml bacitracin. The

final concentrations of radiolabeled and unlabeled IGF were 0.037 nM and 171 nM, respectively. The duration, temperature, pH, and protein concentration used during the binding assay (pH 8.0, 23°C, 3 hrs) were based on previous optimization work using bovine and embryonic chicken (E-15) vitreous humor. The binding reaction was terminated by adding 100 µl of ice cold bovine γ-globulin (3.0 mg/ml), then 300 µl of cold polyethylene glycol (25%; PEG-8000). Following centrifugation (2000 x g, 4°C, 15 min) and aspiration, the surface of the pellet was washed with 300 µl of 12.5% cold PEG. This assay termination procedure precipitates approximately 10% of the free IGF-I and approximately 15% of the free IGF-II. These values were excluded from the analysis. The bound IGF was counted in an LKB gamma counter. Non-specific binding was defined as the binding persisting in the presence of a 4,600-fold excess of unlabeled homologous ligand. Non-specific binding was subtracted from the total binding to yield specific binding. The ratio of specifically bound IGF to free IGF (B/F) was expressed per 50 µl volume of vitreous humor or serum. To assure binding assay linearity, all samples were diluted such that the total binding was 10% or less.

Vitreal and serum IGF-I and IGF-II binding levels were assayed at embryonic days 6, 9, 12, 15 and 19. In addition, binding assays were conducted on samples obtained at 2 and 7 days and 2, 3, 6, and 12 weeks after hatching. At each developmental stage, the level of IGF-I and II binding was assayed in at least two independently-prepared sample pools. The level of binding in each pool was assayed two to four

times in duplicate.

Affinity crosslinking with ^{125}I-IGF-I or IGF-II

^{125}I-IGF-I or IGF-II was crosslinked to vitreal and serum IGFBPs by incubating 100 μl samples (vitreous humor undiluted and serum diluted 1:8) with ^{125}I-IGF (final ^{125}I-IGF concentration = 0.23 nM) for three hours at 23°C. Crosslinking of ^{125}I-IGF to the IGFBPs was achieved by addition of an equal volume of disuccinimidyl suberate (DSS) (0.72 mg/ml) in DMSO to the samples, followed by incubatin for 30 min at 4°C. The crosslinking reaction was terminated by adding a stopping solution containing Tris and EDTA (final concentration Tris= 20.0 mM; EDTA 2.0 mM, pH 6.8). To reduce interference in the radiographs due to the non-specific binding of ^{125}I-IGF to BSA, radiolabeled IGF was prepared in KRP (pH 8.0) with either no BSA or 0.1% BSA. The samples were diluted with reducing sample buffer containing 2.0% SDS, 40.0 mM HEPES (pH 7.0), 10.0% glycerol, 5.0% β-mercaptoethanol and 0.1 mg/ml bromphenol blue and heated to 100°C for 10 min Fifty microliters of the diluted sample was subjected to 12.0% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Tris/Glycine running buffer. Molecular weights were determined using pre-stained molecular weight markers (Amersham) in reducing SDS-PAGE sample buffer. Slab gels (thickness = 1.5 mm) were run at 7.5 mA (constant current) for 12-18 hrs and then at 35.0 mA for 2-3 hrs. The crosslinked ^{125}I-IGF-IGFBP complexes were visualized using Kodak X-AR5 film and a Dupont-Cronex intensifying screen at -

70°C. Crosslinking studies were conducted on samples from each of the developmental stages that had been examined with the binding assay. At each developmental stage, crosslinking experiments were conducted with samples from at least two independently-prepared pools of vitreous humor and serum. Crosslinked samples were run on duplicate gels with consecutive developmental stages in adjacent lanes. When the number of developmental stages to be run exceeded the capacity of the gel, the remaining stages were run on a second gel. The inter-gel variation was monitored by including many of the same samples on both gels.

Western ligand blots

Vitreal and serum samples were subjected to western ligand blotting following a modification of a previously published technique (Hossenlopp et al., 1990). Briefly, samples were subjected to non-reducing SDS gel electrophoresis, transferred to nitrocellulose, and probed with either ^{125}I-IGF-I or ^{125}I-IGF-II. Because of its high protein content, serum was diluted 1:8 prior to the addition of 3x SDS-PAGE sample buffer. Protein was transferred from the gel to 0.2 μm nitrocellulose using either a Biorad Trans-Blot (semi-dry) transfer system (30 min, 60 volts), or a Novex (immersion) transfer system (120 min, 25 volts). The semi-dry buffer consisted of Tris-HCl (25 mM) and glycine (192mM) in 20% methanol. The immersion system transfer buffer contained 10mM sodium borate and 40mM boric acid in 20% methanol. The two transfer systems gave comparable results. Following

transfer, the blot was incubated for 30 min at 4°C in a Tris-buffered saline (TBS) solution (TBS = Tris (10.0 mM), NaCl (150.0 mM), sodium azide (0.5 mg/ml); pH 7.4) with 3.0% (V/V) NP-40. Subsequently, the blot was blocked by incubation (2 hr; 4°C) in TBS with 5.0% BSA. Binding of ^{125}I-IGF-I or II (final concentration = 17.0 pM) to the blot was conducted for 16-20 hrs at 4°C on a rocking platform in TBS with 1.0% BSA and 0.1% Tween-20. To terminate binding, the incubation solution was removed and the blot washed 2 x 20 min on a rocker platform at 4°C with TBS buffer containing 0.1% Tween-20, followed by a 2 x 20 min washes in TBS buffer without Tween-20. The western ligand blot experiments were repeated at least three times using at least two independently-prepared pools of vitreous humor and serum samples. Samples were run on duplicate gels with consecutive developmental stages on juxtaposed lanes as described above.

The affinity of individual vitreal and serum IGFBPs for IGF-I and II was assayed by incubating individual lanes of a transfer blot in ^{125}I-IGF-II with various concentrations of unlabeled IGF-I and II (IGF-II homologous and heterologous competition-inhibition studies). To determine whether the changes in IGF binding activity observed during development represent changes in binding affinity or binding capacity, the competition-inhibition studies were conducted at two developmental stages markedly differing in binding activity. In the case of the vitreous humor, these two stages examined were embryonic days 6 and 19. For the serum, samples from one and three week post-hatching chickens were examined. IGFBPs

were visualized by autoradiography as described above.

Immunoprecipitation

IGFBP-2 antiserum was collected from New Zealand rabbits that were previously immunized with 200 μg of a 20 amino acid synthetic multiple antigenic peptide (Applied Biosystems, Foster City, CA). The peptide sequence was derived from a region near the carboxyl-terminal sequence of human IGFBP-2 (single letter amino acid code: PECHLFYNEQQEARGVHTGR). This antiserum, designated h-C20-IGFBP-2, precipitates a 30-34 kDa IGFBP present in extracellular fluids from a number of different species including human, monkey bovine, rabbit and chicken. Extracellular fluids from which a specific 30-34 kDa IGFBP has been precipitated include serum, vitreous humor, aqueous humor, CSF and amniotic fluid. The antiserum has also been shown to precipitate a 30-34 kDa IGFBP from conditioned media from retinal pigment epithelial cells and retinoblastoma cells. The antiserum is specific, as larger (e.g., 46 kDa) and smaller (e.g., 28 kDa) IGFBPs are not immunoprecipitated.

Immunoprecipitation experiments were conducted by incubating 450 μl of chicken vitreous humor (undiluted) or serum (diluted 1:8) from 2 day post-hatching chickens overnight at 25°C with 50 μl either h-C20-IGFBP-2 (final dilution 1:5) or pre-immune serum (final dilution 1:5). Following this incubation, a protein-A-Sepharose suspension (Pharmacia) was added, and the mixture incubated for an additional 2 hours at 25°C. The protein-A-antibody-antigen

mixture was transferred to an ice bath for 60 min and then centrifuged for 5 min at 12,000 x g. The supernatant was aspirated and the pellet washed three times with TBS. The immunoprecipitated IGFBPs were disassociated from the protein-A-antibody complex by boiling the pellet for 5 min in non-reducing sample buffer. Following centrifugation at 12,000 x g for 5 min the supernatant was electrophoresed, transferred to nitrocellulose and probed with ^{125}I-IGF-II as previously described.

Deglycosylation Studies

Samples of chicken vitreous humor and serum (3 week post hatch) were treated with various deglycosylating enzymes either singly or in combination. The enzymes employed were: 1) N-Glycanase™ alone; 2) neuraminidase alone; or 3) O-Glycanase™ with neuraminidase. N-Glycanase™ [Peptide-N4-(N-acetyl-glucosaminyl)] asparagine amidase and recombinant O-Glycanase™ [endo α-N-acetylgalactosaminidase] were purchased from Genzyme (Boston, MA). Affinity purified Type X neuraminidase was purchased from Sigma Chemical Co. (St. Louis, MO). The reason for treating with both neuraminidase and O-glycanase is that the activity of O-Glycanase is inhibited by the presence of sialic acid side chains on the core oligosaccharide (Genzyme). Experimentally, equal amounts (approximately 20 μg) of vitreal and serum protein (vitreous humor = 20 μl; serum 2.5 μl) were incubated overnight (37°C) with either 4.4 mU of O-Glycanase, 2.2 U of N-Glycanase or 370 mU neuraminidase. Control experiments omitted the enzymes.

After the incubation, the reaction was stopped by adding non-reducing sample buffer and boiling for 10 min. This material was then subjected to western ligand blotting as described above. The presence of glycosylated side chains on IGFBPs was detected by an enzyme-induced reduction in molecular weight.

RNA Extraction and Northern Blot Analysis

Total RNA was isolated from tissues at embryonic day 8, 12, 15, 18 and post-hatching day 2 using a phenol/guanididium hydrochloride extraction technique (RNAzol™, Cinna/Biotecx Laboratories, Friendswood, Texas). RNA was quantified spectrophotometrically at 260 nM using a Beckman model DU-30 spectrophotometer. Approximately five μg of total RNA from various tissues was electrophoresed on a 1% agarose formaldehyde gel at 25 volts for 3 hours. Following electrophoresis, the gel was photographed and soaked in 20X SSC for 15 min to remove excess formaldehyde. The gel was then transferred to a nylon membrane (Nytran™, Schleicher and Schuell) using a Vacuum Gene™ transfer apparatus (Pharmacia). Following the transfer, the blot was washed for 5 min in 20X SSC, blotted damp dry and crosslinked using a UV Stratalinker (Stratagene). Northern blots were hybridized with a ^{32}P-labeled 154 bp PCR probe from exon 2 (described below). Blots were hybridized at 42°C in a 50% formamide solution containing 1x Denhardts, 5x SSC, 1.0% SDS and 10% dextran sulfate for 24 hours. Following hybridization, blots were washed twice at room temperature for 5 min in 1x SSC and 1.0% SDS and then twice at 55°C in 0.1x SSC and 0.01% SDS before auto

radiographic exposure. Autoradiography was performed using X-Omat™ film (Kodak) with an intensifying screen at -70 °C. Individual loading variation was assessed using two techniques. In the first technique, the ethidium bromide gels were photographed and the negative image was digitized. In the second technique, northern blots were stripped and re-probed with an 18S ribosomal PCR probe. Both ethidium bromide staining and reprobing blots with the 18S probe were found to produce similar results. Molecular weight estimation of the hybridizing band was obtained by comparison with the migration of RNA molecular weight standards (Bethesda Research Laboratory). Negatives of ethidium bromide stained gels and northern blot autoradiographs were analyzed using a model 620 video densitometer and 1-D Analyst software (Biorad, Hercules, CA).

Preparation of PCR probe for library screening and northern blots

Nucleic acid sequences from rat, human and bovine IGFBP-2 were obtained from Genbank and aligned using a nucleic acid analysis program (DNAStar,Inc., Madison, WI, U.S.A.). Primers were chosen encompassing a conserved region of 154 bp in exon-2. The sequences of the primers was 5'GAGCAGCACCGGCAGATG 3' and 5'TACAGGCCATGCTTGTCACA3' respectively. cDNA was prepared from several embryonic day 15 tissues (Wilkie and Simon, 1991) and was amplified using a PHC-2 Dri-Block™ thermal cycler (Techne).

The reaction mixture contained approximately 100 ng of

cDNA, 25 picomole of each primer, 200 uM dNTP's, 10 mM Tris (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$ and 2.5 units of Taq polymerase (Stratagene). Amplification was performed in one cycle of 2 min at 95°C, followed by 30 cycles of a 45 sec. denaturation step at 95°C, a 1 min annealing step at 50°C and a 1 min extension at 72°C, with a final extension step of 5 min. A single 154 bp PCR product was obtained, sequenced and confirmed to be similar to mammalian IGFBP-2. The 154 bp PCR product was labeled with ^{32}P-dCTP to a specific activity of approximately 5 x 10^9 cpm/μg using a PCR-labeling technique (Schowalter and Sommer, 1989). This probe was used for screening the cDNA library as well as for northern blot analysis. The 18S probe was prepared by selecting primers in a conserved region of several mammalian cDNAs and performing PCR on retina cDNA. The 18S probe was labeled in essentially the same way as the IGFBP-2 probe. The sequence of the 18S primers are: 5'CAAAGATTAAGCCATGCATGTC3' and 5'TCGGCTCGAGGTTATCTAGAGT 3'.

Complementary DNA library preparation and screening

An embryonic 18 day (E-18) chicken retina cDNA library was constructed in Lambda Zap (Stratagene, La Jolla, CA, U.S.A). Construction followed the directions of the manufacturer except that 50 μg of total RNA was used in place of the recommended 5.0 μg of poly A mRNA. Approximately 50,000 independent phage cDNA clones were screened. Hybridization of the colony lifts was performed at 42°C for 18 to 24 hrs in a solution of 50% formamide, 5X SSC, 1X

Denhardt's, 1.0% SDS and 10% dextran sulfate and approximately 10^6 cpm/ml of the ^{32}P-labeled chicken embryo IGFBP-2 PCR probe. Positive clones were isolated, excised and plasmid DNA was purified (Qiagen Inc., Chatsworth, CA, U.S.A.) and sequenced using a fluorescent Taq DyeDeoxy Terminator Sequencing reaction (Applied Biosystems, Foster City, CA, U.S.A.). Sequence reactions were analyzed on a 6% acrylamide sequencing gel using an Applied Biosystems model 373A fluorescent sequencer.

5' Rapid Amplification of cDNA Ends (5' RACE)

The 5' Amplifinder™ RACE System (Clontech, Palo Alto, CA) was utilized to obtain 5' sequence. Briefly, 1 ug of poly A+ RNA from adult chicken brain (Clontech, Palo Alto, CA) was reverse transcribed using a specific primer (GSP1: 5'ATCACCGCCATCTCCTTCATG 3'). Contaminating RNA was removed by alkaline hydrolysis and the cDNA was purified by binding to a silica matrix (Genobind™,Clontech, Palo Alto, Calif.). A single-stranded oligonucleotide (Amplifinder Anchor: 5'P-CACGAATTCACTATCGATTCTGG-AACCTTCAGAGG-NH3') was ligated to the 3'end of the cDNA using T_4 RNA ligase. Following an overnight ligation at 25°C, a 1.0 ul aliquot of the ligation mixture was added to a 50 ul PCR reaction containing: 25 pmoles of a nested specific primer (GSP2: 5'TCAGACCGGTCATCACCGTTG 3') and 25 pmoles of Amplifinder Anchor primer: 5'CTGGTTCGGCCCACCTCTGAAG-GTTCCAGAATCGATAG3'. PCR amplification was performed in a PHC-2 Dri-Block™ thermal cycler (Techne). The reaction mixture contained 100 ng of

cDNA, 25 picomole of each primer, 200 uM dNTP's, 10 mM Tris (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$ and 2.5 units of Taq polymerase (Statagene). Amplification was performed after 1 min at 95°C, followed by 30 cycles of a 45 sec. denaturation step at 95°C, a 1 min annealing step at 60°C and a 1 min extension at 72°C, with a final extension step of 5 min. PCR products were analyzed by adding 2.0 ul of sample buffer (15% Ficoll, 0.25% Bromophenol Blue, 5.0mM K_2HPO_4) to 10 ul of PCR product, followed by electrophoresis on a 6% acrylamide Tris Borate EDTA (TBE) gel (Novex, San Diego, Calif.). PCR products were cloned into a pGEM™-T Vector (Promega, Madison, Wisc.), transformed using JM109 competent cells and plated onto agar plates containing 50ug/ml of ampicillin, 40μg/ml of X-gal and 40μg/ml of IPTG. White colonies were selected and sequenced.

Genomic Cosmid Library Screening

Initial screening of 100,000 clones from a chicken genomic cosmid library (pWE 15, Clontech) using the 154 bp PCR product from exon 2 yielded several clones containing exons 2, 3 and 4, but lacking exon 1. Therefore, a 225 bp RACE product containing a small portion of exon-1 was used for screening. Screening of another 100,000 clones yielded two clones both containing exon-1, as well as a portion of intron 1 and 5' upstream sequence. Clones were restriction digested, and Southern blots were probed with the exon-1 specific probe. Hybridizing bands were excised, subcloned into KS+ Bluescript and sequenced in both directions using fluorescent sequencing

as previously described.

Genomic Southern blot

Samples containing 10 μg of high molecular weight chicken DNA (male broiler, Clontech) and 300 ng of cosmid DNA were digested with selected restriction enzymes for 16 hrs at 30°C. The fragments were separated on a 0.7% (w/v) agarose (0.5X TAE) gel at 1 V/cm, stained in ethidium bromide (0.5 g/ml), photographed and transferred to a nylon membrane as described for northern blots. Following the transfer, the blot was hybridized overnight at 42°C with a ^{32}P-labeled PCR product from exon 1. The blot was then stripped and re-probed with an exon 2 specific probe. Overlap between clones was confirmed by PCR, sequencing and multiple Southern blots.

***In Situ* Hybridization**

The *in situ* hybridization technique is based on a *in situ* hybridization protocol by Bondy *et al.*, 1990. In this case, whole eyes from embryonic day 6 and day 12 chickens were fixed in 10% buffered formalin a minimum of 24 hours. Tissues were embedded in paraffin and 5-10 μm sections were cut and placed on silane coated slides. Following removal of the paraffin with xylene and rehydration through graded ethanols, slides were incubated with proteinase K (1μg/ml) for 30 min at 37°C, then treated with 0.1 M triethanolamine for 10 min followed by 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min at room temperature, washed and dehydrated. Hybridization was performed with 10^7 DPM/ml or 50ng/ml of the

cRNA probe (described below) in a hybridization buffer containing 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 500 μg tRNA/ml, 10% dextran sulfate, 10 mM dithiothreitol and 0.02% each of BSA, Ficoll and polyvinylpyrrollidone.

Following incubation at 55 °C for 14-16 hours in a humidified chamber, slides were washed several times in 4X SSC followed by dehydration through a series of graded ethanols and immersed in 0.3 M NaCl, 50% formamide, 20mM Tris-HCl and 1 mM EDTA at 60 °C for 10 min. Sections were then treated with RNase-A (20 μg/ml) for 30 min at room temperature, followed by a 15 min wash in 0.1X SSC at 55 °C. After a final dehydration, sections were air dried and coated with Kodak NTB3 nuclear emulsion and stored with desiccant for 6 to 12 days. Slides were then developed with Dektol and stained with hematoxylin and eosin for microscopic evaluation.

^{35}S-labeled cRNA probes were synthesized in 10 μl reactions containing 250 μCi ^{35}S-CTP and 250 μCi ^{53}SUTP, 10 mM NaCl, 6 mM $MgCl_2$, 40 mM Tris (pH 7.5), 2 mM spermidine, 10 mM dithiothreitol, 500 μM each of unlabeled ATP and GTP, 25 μM each of unlabeled UTP and CTP, 500 ng linearized template, 15 U of the appropriate polymerase (T3 for the control sense probe; T7 for the specific IGFBP-2 antisense probe) and 15 U of RNasin. The reaction was incubated at 42 °C for 1 hour, after which the DNA template was removed by digestion with DNase-I at 37 °C for 10 min. Labeled cRNA was purified by the addition of 50 μg yeast tRNA, phenol- chloro-form extraction, chloroform extraction and ethanol precipitation. The pellet

was re-suspended in 200 μl 0.2% sodium dodecyl sulfate, 2mM EDTA and 0.3 M ammonium acetate (pH 5.2) and precipitated with cold ethanol. The average specific activity of probes generated in this protocol was 2-3 X 10^8 dpm/μg.

CHAPTER III

Local Synthesis and Independent Developmental Regulation of Avian Vitreal Insulin-like Growth Factor Binding Proteins: A Model for Independent Developmental Regulation in Extravascular and Vascular Compartments

Introduction

The vitreous humor, a specialized ocular extracellular fluid protected from serum by the blood-ocular barrier (Cunha-Vaz, 1979; Latker and Beebe, 1984), has been shown to contain several high affinity IGFBPs (Grant *et al.*, 1986; Ocrant *et al.*, 1991; Waldbillig *et al.*, 1991). While the origin of vitreal IGFBPs remains to be determined, mRNA for one of the major vitreal IGFBPs, IGFBP-2, has been identified in several ocular tissues (Agarwal *et al.*, 1991; Arnold *et al.*, 1993), suggesting local ocular synthesis.

In order to determine whether vitreal IGFBPs form part of an autonomous ocular IGFBP system, vitreal and serum IGFBPs were examined for similarities and differences in their pattern of developmental regulation, structure and

glycosylation.

Results

^{125}I-IGF-I and -II binding assay

The upper panel of Fig. 5 shows the levels (mean ± SEM) of vitreal and serum ^{125}I-IGF-I binding activity at various stages of development (note the interrupted ordinate in this figure). Vitreal IGF-I binding is highest on embryonic day 6 and decreases 10 fold between E-6 and E-19 (mean ± SEM B/F; E-6= 0.22 ± 0.019; E-19= 0.022 ± 0.003). In contrast, serum IGF-I binding increases more than two-fold during the same period, from B/F= 0.380 ± 0.056 on E-6 to B/F= 0.89 ± 0.18 on E-19.

Within two days of hatching, there is a two-fold increase in IGF-I binding in both the vitreous humor and serum. In the interval between post-hatching day 2 (PH-D2) and week 12 (PH-W12), IGF-I binding in the vitreous humor remains relatively stable while serum IGF-I binding again increases approximately two-fold (PH-D2 serum B/F= 2.4 ± 0.35; PH-W12 serum B/F= 4.1 ± 0.14). The lower panel of Fig. 5 shows that IGF-II binding in the vitreous humor and serum has a developmental pattern that is very similar to that observed for IGF-I binding. However, at all developmental stages, IGF-II binding is 2-3 fold higher than IGF-I binding.

Affinity Crosslinking

In order to examine the structural basis of IGF

binding, vitreal and serum IGFBPs from various developmental stages were crosslinked to ^{125}I-IGF-I and ^{125}I-IGF-II. Fig. 6 shows the crosslinked vitreal and serum ^{125}I-IGF-IGFBPs observed in two day post-hatching chickens under reducing SDS-PAGE conditions. The two left hand lanes show that IGF-I affinity crosslinked vitreal and serum IGFBPS appear as single radiographic bands. It can be seen, however, that the vitreal IGFBPs have a slightly higher molecular weight and a broader radiographic profile than their serum counterparts (vitreous humor= 40-47; serum= 42-44 kDa). The two right hand lanes of Fig. 6 show that when IGF-II, instead of IGF-I, is used for affinity-crosslinking, there are again differences in the vitreal and serum IGFBPs that are similar to those seen with IGF-I. One of these differences is that IGF-II affinity crosslinked IGFBPs in serum appear as two distinct bands (39 and 43 kDa), while the crosslinked vitreal IGFBPs appear as a single broad band (43-47 kDa). Again, vitreal IGFBPs appear to be generally larger than serum IGFBPs. The micro-heterogeneity observed in IGF-I and II affinity crosslinked vitreal and serum IGFBPs shown for the PH-D2 chickens is also observed at the other developmental stages (data not shown). Developmental trends in the level of vitreal and serum IGF-I and II binding activity (observed in binding assays), was also observed in affinity crosslinked samples (data not shown).

^{125}I-IGF-II western ligand blots

Vitreous humor and serum samples were electrophoresed under non-reducing conditions, blotted to nitrocellulose and

probed with ^{125}I-IGF-II. The developmental pattern of vitreal and serum IGFBPs did not vary when either ^{125}I-IGF-I or ^{125}I-IGF-II was used. However, since the ^{125}I-IGF-II signal-to-noise ratio was superior to that of IGF-I (data not shown), subsequent blots were probed with ^{125}I-IGF-II.

The western ligand blot technique reveals a complex population of vitreal and serum IGFBPs not visualized by affinity crosslinking or IGF binding assay. In particular, they show a compartment-specific, developmental pattern, with unique IGFBPs appearing and disappearing at specific developmental stages. Fig. 7 shows a representative ^{125}I-IGF-II western ligand blot of vitreal and serum IGFBPs at two stages of embryonic development (E-6 and E-15). On embryonic day 6 (left two lanes of Fig. 7), the vitreous humor contains a 28 kDa band not detected in the serum even with extended radiographic exposures. There are also differences in vitreal and serum IGFBPs at embryonic day 15 (right two lanes of Fig. 7). For example, vitreous humor at E15 contains a small IGFBP (24 kDa) not detected in the serum even with extended radiographic exposures. Likewise, at E-15 serum contains 42 and 28 kDa IGFBPs not present in the vitreous humor. Western ligand blots of vitreous humor and serum at earlier and later embryonic stages also exhibit differences in IGFBPs (data not shown). Only the 33 kDa IGFBP appears in the vitreous humor and serum at all embryonic stages.

Western ligand blots of vitreous and serum at post-hatching week 1 and 3, also show differences in the pattern of IGFBPs (Fig. 8). At one week post-hatching, the serum

contains a 70 kDa band not detected in the vitreous humor. The intensity of the serum 70 and 42 kDa IGFBPs markedly increase between post-hatching week one and three. In contrast, at three weeks post hatching, there is only a trace of the 42 kDa band in the vitreous humor. Another difference in vitreal and serum IGFBPs is that the vitreal 28 kDa IGFBP increases significantly between post-hatching week 1 and 3 while the serum analogue only slightly increases in this period. Similar differences were also observed at post-hatching weeks 6 and 12 (data not shown).

IGF-II western ligand blots used in homologous and heterologous competition/inhibition studies revealed that at two embryonic days (E-6 and E-19) and two post-hatching (PH-1W and PH-3W) stages, vitreal and serum IGFBPs have a higher affinity for IGF-II than IGF-I (data not shown). ^{125}I-IGF-II binding to vitreal and serum ligand blots was 50% inhibited by approximately 0.1 nM unlabeled IGF-II or 1.0 nM unlabeled IGF-I. The apparent affinity of individual vitreal or serum IGFBPs did not appreciably vary across the four developmental stages examined.

Immunoprecipitation

Although vitreous and serum exhibit different patterns of IGFBP developmental regulation and contain IGFBPs of different sizes, a 33 kDa IGFBP band is common to both compartments throughout development. In order to determine if the 33 kDa IGFBP represents the avian homologue of IGFBP-2, immunopreciptation was performed using an antiserum produced

against a 20 amino acid sequence near the carboxyl-terminus of human IGFBP-2. Vitreous humor and serum from post hatching day 2 chickens were immunoprecipitated with the IGFBP-2 antiserum and examined by the western ligand blot technique (Fig. 9). It is apparent that both the vitreal and serum 33 kDa IGFBPs are precipitated by IGFBP-2 antiserum. The immunoprecipitation is specific since non-immune rabbit serum is ineffective in precipitating these IGFBPs (Fig. 9, see lanes labeled N.S.). Further evidence that the immunoprecipitation of the vitreal and serum 33 kDa IGFBP is specific is that the 28 kDa IGFBP present in these fluids is not immunoprecipitated by this antiserum. The faint 31 kDa band observed in the immunoprecipitated vitreal lane represents a rabbit IGFBP that contaminates the immunoprecipitate. The contaminating rabbit serum IGFBP is also observed when immunoprecipitation studies are carried out in the absence of vitreous and serum (data not shown).

Deglycosylation

To examine further the similarities and differences between the vitreal and serum 33 kDa IGFBPs, their glycosylation patterns were examined. Samples of vitreous humor and serum from post-hatching week 3 were treated with various deglycosylating enzymes: N-Glycanase alone (cleaves N-linked oligosaccharides); neuraminidase alone (cleaves sialic acid residues); and O-Glycanase plus neuraminidase (cleaves O-linked oligosaccharides). Samples were then examined by the western ligand blot technique.

Fig. 10 shows that the 33 kDa IGFBP found in the vitreous and serum is differentially glycosylated in the two compartments. Specifically, the apparent molecular weight of the serum, but not the vitreal, 33 kDa IGFBP is reduced following deglycosylation with neuraminidase alone or neuraminidase added to O-Glycanase. The apparent molecular weight of the de-sialated 33 kDa IGFBP is approximately 28 kDa (see lane labeled Neura). When O-Glycanase is added with the neuraminidase (see lane labeled O-Gly), the apparent molecular weight is slightly, but reliably, further reduced. Attempts to determine whether the vitreal and serum 33 kDa IGFBP differ in N-linked glycosylation patterns were complicated by the superimposition of the 42 kDa IGFBP over the 33 kDa position following deglycosylation.

In addition to differences in the glycosylation of the 33 kDa IGFBP, serum and vitreous humor also differ in the glycosylation of the 42 kDa IGFBP. In serum, N-Glycanase decreases the size of the 42 kDa IGFBP and increases its binding activity. The vitreal 42 kDa is also reduced in size by N-Glycanase, but there is no indication that its binding activity is increased. The N-Glycanase-induced changes in binding activity of the serum 42 kDa IGFBP were observed in two independent experiments.

Discussion

The results of this study demonstrate the presence of specific, high affinity IGFBPs in embryonic and post-hatching

chicken vitreous humor and serum. Importantly, the IGFBPs in the two compartments exhibit differential developmental regulation. IGF binding in the vitreous humor is highest on embryonic day 6, decreases on embryonic day 9 and then remains relatively stable throughout the remainder of embryonic and post-hatching development. In contrast, serum IGF binding steadily increases throughout embryonic and post-hatching development. While the binding protein assay is limited to measuring only IGFBPs that have free binding sites, similar developmental trends in IGFBP levels were observed in western ligand blot studies (data not shown). This indicates that most of the IGFBPs in avian vitreous and serum contain free binding sites. Data from ^{125}I-IGF-II western ligand blot competition/inhibition studies indicate that the affinity for IGF-II does not vary across development or among the different IGFBPs (data not shown). Thus, it appears that the developmental variation in binding activity is the result of changes in the relative abundance of vitreal and serum IGFBPs.

The maintenance of relatively constant levels of vitreal IGF binding activity during stages of embryonic eye growth and vitreal volume expansion suggests that IGFBPs are actively being added to the vitreous humor in a way that offsets the dilution that would otherwise occur. In contrast to reports of decreases in vitreal IGFBPs in neonatal rats (Ocrant *et al.*, 1991), the post-hatching chicken exhibits stable vitreal IGFBP levels throughout adulthood suggesting either a lack of turnover, or more likely, an equilibrium between synthesis and degradation.

In distinction to the multiple IGFBPs revealed by western ligand blots, affinity-crosslinking studies show only single, albeit broad, bands. The difference between crosslinking and ligand blot techniques suggests either that some IGFBPs are not crosslinkable or are differentially saturated with endogenous ligand. Although affinity crosslinking studies may not reveal all IGFBPs, the finding that the crosslinked vitreal and serum IGFBPs exhibit differences in mobility suggests that vitreal and serum IGFBPs are structurally distinct.

Further evidence that the vitreal and serum IGFBP systems are independent of each other comes from western ligand blot studies showing that the vitreous contains a low molecular weight IGFBP (24 kDa) not detected in the serum at any stage. Additionally, these blots show that post-hatching serum contains a high molecular weight IGFBP (70 kDa) not present in the vitreous. The presence of the blood-ocular barrier makes it unlikely that the low molecular weight vitreal IGFBPs are proteolytic fragments of larger serum IGFBPs.

The pattern of IGFBPs observed using western ligand blot analysis of early post-hatching chicken serum resembles that previously reported (Armstrong *et al.*, 1989). However, their study did not observe the 70 kDa serum IGFBP reported here. This apparent discrepancy may be related to strain differences, or possibly, differences in the transfer efficiency of the IGFBPs from the gels. The finding that serum IGFBP levels steadily increase throughout post-hatching

stages of development is compatible with other observations in both chickens (Lee *et al.*, 1989) and post-natal mammals (Hardouin *et al.*, 1989). The large increase in the serum 42 kDa IGFBP seen after hatching may be the result of the onset of the influences of growth hormone which has been shown to increase in serum following hatching (Kikuchi *et al.*, 1991). Similarities between the chicken 42 kDa IGFBP and mammalian IGFBP-3 in terms of molecular weight (Conover *et al.*, 1990), glycosylation (Baxter and Martin, 1989) and possible growth hormone sensitivity (Baxter and Martin, 1986), suggest that this IGFBP may be the avian analogue of mammalian IGFBP-3.

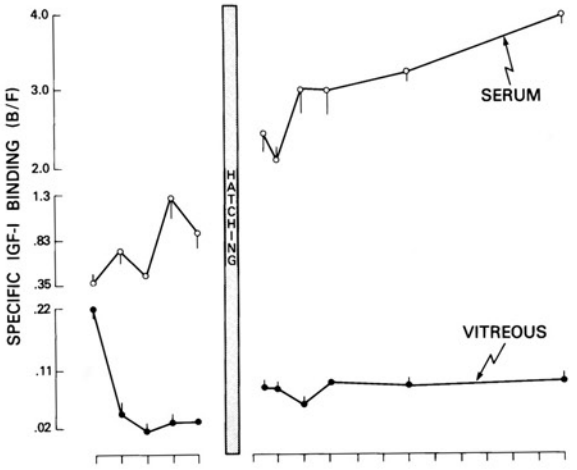
Since IGFBP-2 antiserum immunoprecipitates both the vitreal and serum 33 kDa IGFBPs, it suggests that these two IGFBPs are structurally similar to each other and to human IGFBP-2. However, although the vitreous humor and serum both contain IGFBP-2, the fact that the vitreal form is not sialated is further evidence for an independent vitreal IGFBP system. It should be pointed out that it is not clear how the vitreal and serum forms of IGFBP-2 can have the same apparent molecular weight, while differing in glycosylation. Possibly, differences in secondary structure cause IGFBPs of different sizes to migrate at the same electrophoretic position. Another possibility, is that the vitreal and serum IGFBP-2 represent products of alternative transcription and/or different post translational modification. In this scheme, the serum IGFBP-2 would have a smaller protein core, but would have glycosylated side chains that offset the decreased protein size.

Another glycosylation difference between vitreal and serum IGFBPs is seen in the 42 kDa IGFBP. Here, treatment with N-glycanase results in an increased binding activity of the serum, but not the vitreal, 42 kDa IGFBP. The increase in the binding activity of the serum 42 kDa IGFBP following N-deglycosylation is reminiscent of similar findings with insulin (Podskalny *et al.*, 1986) and prolactin receptors (Lascols *et al.*, 1989).

In summary, the finding that in vitreous humor, individual IGFBPs exhibit glycosylation and developmental regulation patterns that are different from those observed for the serum IGFBPs suggests the presence of an autonomous ocular IGFBP system.

Fig. 5 **^{125}I-IGF-I and -II binding assay of vitreous humor and serum.** ^{125}I-IGF-I (upper panel) and ^{125}I-IGF-II (lower panel) specific binding (B/F) in developing chicken vitreous humor (●) and serum (○). Binding is expressed per 50 ul of fluid. Each point represents the mean ± SEM for a minimum of two assays conducted in duplicate. The number of chickens used at each time point varied with age as described in *Materials and Methods*.

IGF-I BINDING ACTIVITY IN DEVELOPING CHICKEN VITREOUS AND SERUM



IGF-II BINDING ACTIVITY IN DEVELOPING CHICKEN VITREOUS AND SERUM

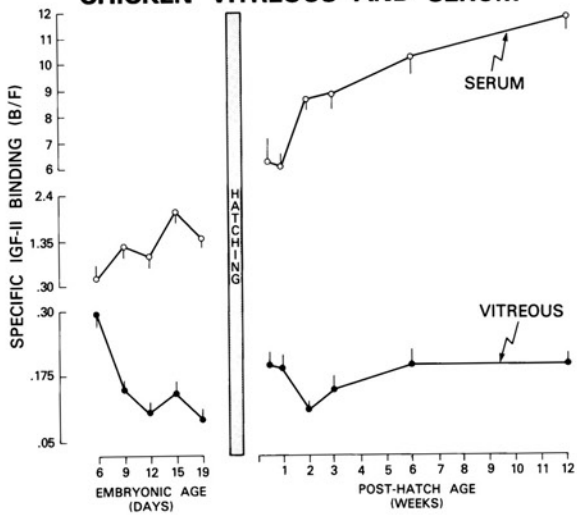


Fig. 6 **^{125}I-IGF-I and -II affinity crosslinking of vitreous humor and serum.** IGF-I (left two lanes) and IGF-II (right two lanes) affinity crosslinking to IGFBPs in vitreous humor and serum of 2 day post-hatching chickens. Fifty ul of vitreous humor or 6 ul of serum was crosslinked to either ^{125}I-IGF-I or ^{125}I-IGF-II using disuccinimidyl suberate (DSS). Samples were subjected to electrophoresis under reducing conditions on a 12.5% polyacrylamide gel and visualized by autoradiography.

IGF AFFINITY CROSSLINKING TO VITREAL AND SERUM IGF-BPs

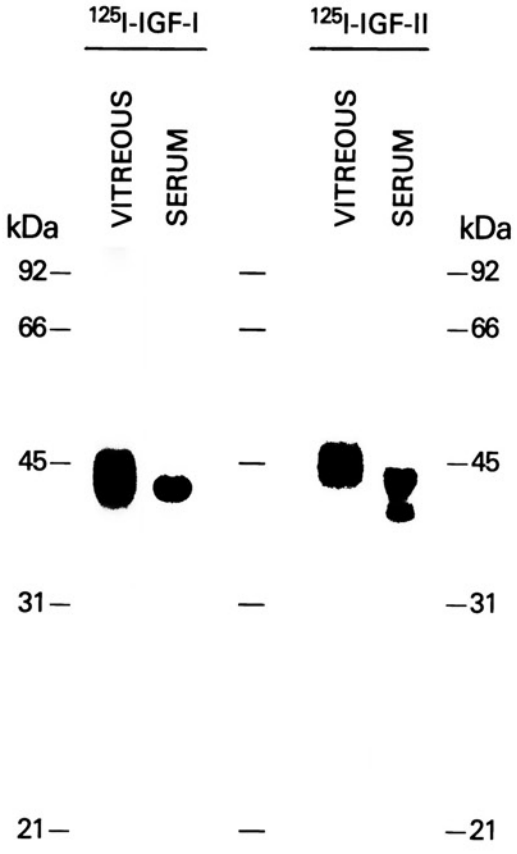


Fig. 7. **Western ligand blot analysis of embryonic vitreous humor and serum.** IGF-II western ligand blots of vitreous humor and serum from chickens at two embryonic stages (day 6 and 15) of development. Fifty ul of vitreous humor or 6 ul of serum were subjected to electrophoresis on a 12.5% gel, transferred to a nitrocellulose membrane, probed with ^{125}I-IGF-II and visualized by autoradiography.

^{125}IGF-II LIGAND BLOT EMBRYONIC CHICKEN

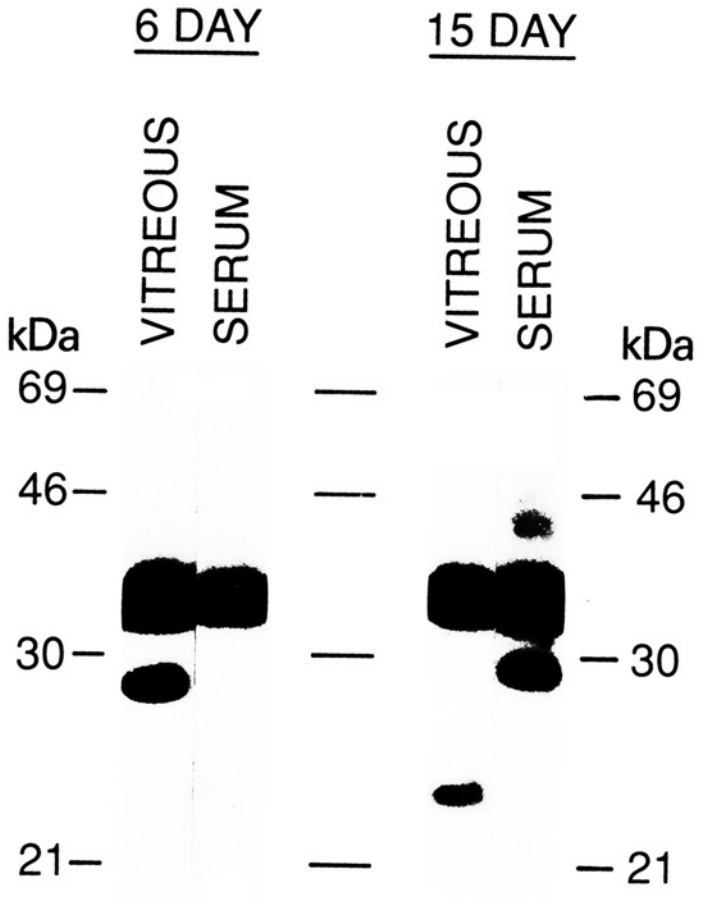


Fig. 8. **Western ligand blot analysis of post-hatching vitreous and serum.** IGF-II western ligand blotting of vitreous and serum from chickens at two post-hatching stages (week 1 and 3). Fifty ul of vitreous humor or 6 ul of serum were subjected to ligand blotting as in Fig. 7.

^{125}IGF-II LIGAND BLOT
POST-HATCHING CHICKEN

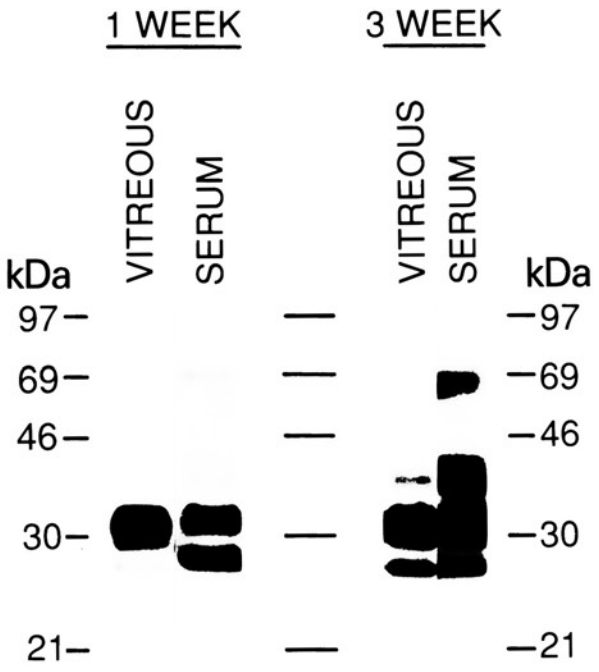


Fig. 9. **Immunoprecipitation of vitreal and serum IGFBP-2.** Immunoprecipitation and IGF-II western ligand blotting of vitreal and serum IGFBPs from 2 day post-hatching chickens. Fifty ul of undiluted IGFBP-2 antiserum (h-C20-IGFBP-2) was incubated with 450 ul of vitreous humor or serum (1:8), precipitated with protein-A and subjected to ^{125}I-IGF-II ligand blotting as described in *Materials and Methods*. Total= non-immunoprecipitated vitreous humor or serum; Non-specific (N.S.)= non-immune rabbit serum immunoprecipitated; Specific= h-C20-IGFBP-2 anti-serum immunoprecipitated.

IGF-BP-2 Immunoprecipitation

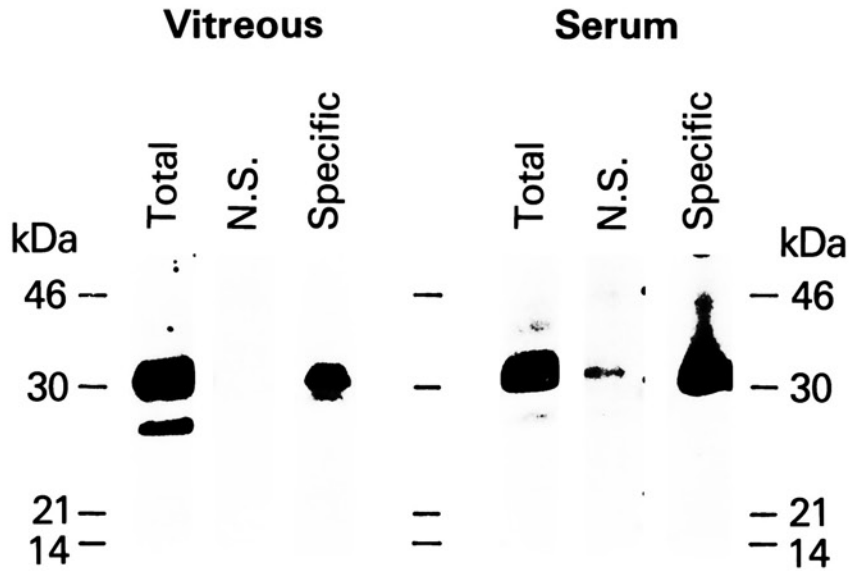
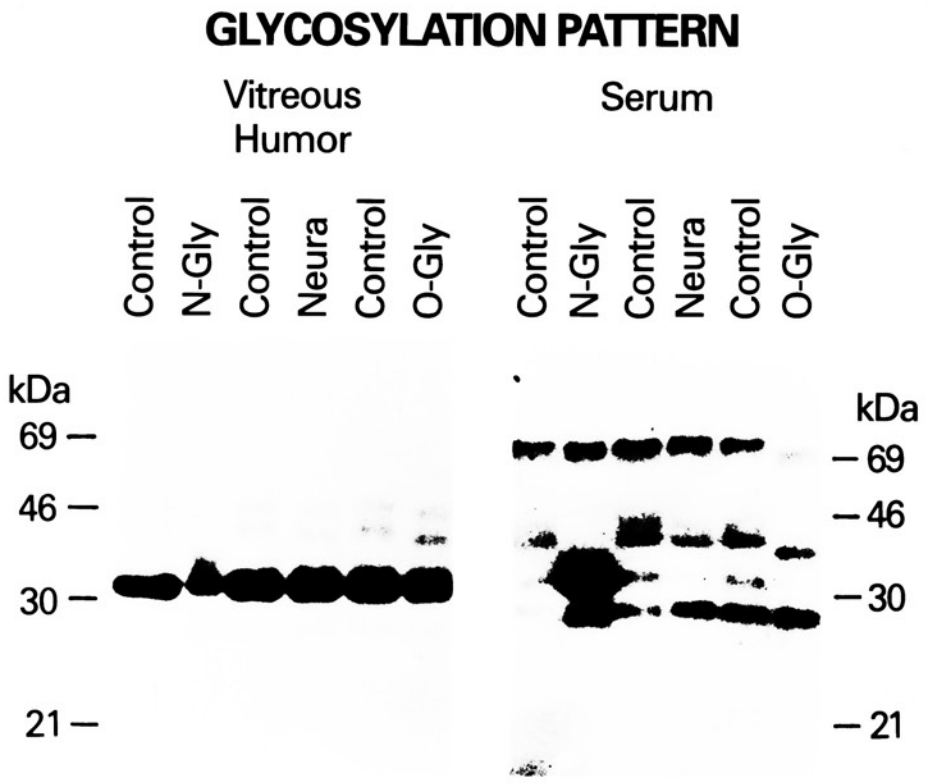


Fig. 10. **Deglycosylation of vitreal and serum IGFBPs.** O- and N-linked deglycosylation of 3 week post-hatching chicken vitreous humor and serum. Twenty ul of vitreous humor or 2.5 ul of serum was treated with N-Glycanase (N-Gly), neuraminidase (Neura) or neuraminidase and O-Glycanase (O-Gly) and then subjected to western ligand blotting as described in *Materials and Methods*. Similar patterns were observed in two independent experiments.


GLYCOSYLATION PATTERN
Vitreous Humor
Serum
Control
N-Gly
Control
Neura
Control
O-Gly
Control
N-Gly
Control
Neura
Control
O-Gly
kDa
69
46
30
21
kDa
69
46
30
21

CHAPTER IV

Cloning, Characterization, and Expression of an Embryonic Chicken cDNA and Gene for Insulin-like Growth Factor Binding Protein-2

Introduction

The results of western ligand blot analysis of chicken embryo vitreous humor, as well as analysis of bovine ocular tissues (Arnold *et al.*, 1993) indicated that IGFBP-2 is the major binding protein in ocular tissues and fluids. Therefore, it was decided to investigate the expression of IGFBP-2 mRNA in ocular tissues during development using both northern blotting and *in situ* hybridization. However, in order to study IGFBP-2 expression, it was first necessary to obtain a suitable IGFBP-2 probe. Since the chicken IGFBP-2 sequence was not known, the published cDNA sequences from the human, rat and cow were aligned and PCR primers were chosen to encompass a 154bp sequence in a conserved region of exon-2 (Fig. 11A). A PCR product was obtained and confirmed by sequencing to be the chicken homolog of mammalian IGFBP-2 (Fig. 11B). This PCR product was used to screen an embryonic day 18 cDNA library in order to obtain a full length cDNA clone that could be used for northern blot analysis and *in situ* hybridization. The results presented below provide a description and comparison of the primary cDNA sequence,

genomic organization and tissue expression of the chicken IGFBP-2 with that of its mammalian counterparts.

Results

Isolation and sequence analysis of an embryonic chicken IGFBP-2 cDNA

Screening of 50,000 recombinant clones from an E18 chicken retina cDNA library yielded a clone (1600 bp) that exhibited approximately 60% identity to mammalian IGFBP-2. Comparison of the chicken cDNA clone sequence with IGFBP-2 sequence from the rat, cow and human revealed that approximately 700 bp of 5′ sequences, including the initiation codon was missing. Therefore, the 5′ RACE technique was used to obtain an additional 300 bp of sequence (Fig. 12, single underlined sequence). Because of difficulties in obtaining a 5′ RACE product that extended to the initiation codon, a chicken genomic library was screened using an exon 1 specific probe. One of the cosmid clones isolated (Clone 1A) was found to contain sequence that matched the 5′ RACE sequence and contained an initiation codon that was preceded by a Kozak consensus sequence (**ccgcc**; Kozak, 1984) (Fig. 12, double underlined sequence). The complete open reading frame of 933 bp contains a 34 amino acid putative hydrophobic signal peptide (Watson, 1984) that is present in all mammalian IGFBP-2s. The mature 275 amino acid protein has a predicted Mr of 33,500, with no potential N-glycosylation sites. Interestingly, the avian IGFBP-2 contains a large 3′

untranslated region (UTR) of approximately 1100 bp, while the average size of the mammalian IGFBP-2 3′ UTR is only approximately 350 bp. Contained within the 3′ UTR is a single ATTTA motif that is thought to regulate RNA stability (Shaw and Kamen, 1986). While no poly A tail was present in our cDNA clone, an additional 40 bp obtained from genomic sequence (clone 2A-1) was found to contain the putative AATAAA polyadenylation signal (Fig. 12, position 2090-2095 bp).

Comparative analysis of the deduced peptide sequence

The alignment of the deduced amino acid sequence of the avian IGFBP-2 with that of several mammalian species is shown in Fig. 13. The avian IGFBP-2 exhibits an overall identity of 71, 68, 68, and 66% to rat, bovine, ovine and human IGFBP-2 cDNAs respectively. All 18 cysteines of the avian IGFBP-2 are conserved in comparison with the mammalian IGFBP-2's. Interestingly, there is a 14 amino acid stretch (Fig. 13, position 18-32) that is absent in the chicken.

The RGD tripeptide sequence, present in all of the mammalian IGFBP-2 sequences is also present in the chicken. However, the putative ATP binding site (GXGXXG) followed by a lysine residue 17 a.a. downstream which is present in the human, bovine and ovine IGFBP-2 sequences is absent in both the chicken and the rat.

Genomic analysis

In order to assess the complexity of the gene and the extent of coverage by our genomic cosmid clones, Southern blot

analysis was performed (Fig 14). Samples of restriction digested chicken genomic and cosmid DNA (clone 9A-1) were separated in 0.75% agarose, blotted and probed with an exon-2 specific fragment. The finding of a single major band in each of the three restriction digested genomic samples suggests that the IGFBP-2 gene is most likely present as a single copy in the avian genome. Hybridizing bands of approximately 14, 13 and 2 kb are observed for genomic DNA digested with EcoRI, BamHI and HindIII, respectively. Both the BamHI and HindIII fragments of cosmid 9A-1 are similar in—size to the corresponding genomic fragments, suggesting that clone 9A-1 encompasses these fragments. Further restriction analysis coupled with PCR and sequencing data indicate that the smaller EcoRI fragment (9 kb as compared to ~14 kb for genomic DNA) of clone 9A-1 is the result of the clone lacking a portion of intron 1 and exon 1 (data not shown).

The basic organization and partial restriction map of the avian IGFBP-2 gene is shown in Fig. 15, along with regions of the gene contained in cosmid clones. The results of restriction mapping, PCR and sequence analysis demonstrates that the entire chicken IGFBP-2 gene of approximately 38 kb, is contained within cosmid clones 1A, 9A-1 and 2A-1. Cosmid 1A contains 5' upstream sequence, exon 1, part of intron 1 and overlaps cosmid 9A-1 by approximately 500 bp in intron 1. Restriction map analysis of 1A and 9A-1 indicates that intron 1 is approximately 32 kb. Although clone 9A-1 overlaps with clone 1A, it is lacking exon 4, which is contained in clone 2A-1.

As is true for the mammalian IGFBP-2 gene, the avian IGFBP-2 gene is organized into 4 exons and 3 introns. The sizes of the exons and introns and the exon/intron border sequence is shown in Fig. 16. All exon/intron boundaries conform to the GT/AG rule (Kozak, 1987). In addition, the avian splice junctions occur at or between the same amino acids as in the rat and human IGFBP-2 genes. With the exception of exon 4 which contain the 3′ UTR, the avian IGFBP-2 exons and introns are similar in size to those of the rat and human. Exon-1 consists of approximately 550 bp of which 441 are part of the open reading frame. Exon-1 is extremely GC rich (85%) as compared to exons 2, 3 and 4 (54%, 60% and 55% respectively). As stated previously, intron-1 is approximately 32 kb in size. Exon 2 is 225 bp and intron 2 is approximately 700 bp. Exon 3 is 141 bp and shows the greatest conservation of all 4 exons with a 98% amino acid identity across all species. Exon 4 contains an unusually long 3′ UTR of ~1100 bp along with 165 bp of coding sequence.

Approximately 700 bp of the 5′ flanking region of the avian IGFBP-2 gene have been sequenced and are shown in Fig. 17. Because the actual transcription start site has yet to be determined, the first base of the initiation codon is numbered +1. No putative TATA or CCAAT consensus sequences are observed in this region. However, several putative promoter elements previously identified in the human, rat and mouse IGFBP-2 gene are present in the chicken in approximately the same locations. A Pan motif (Nelson *et al.*, 1990) is located at position -32 to -26 . Three Sp1 sites (Faisst and Meyer,

1992) are present at positions: -160 to -157, -247 to -242 and -323 to -318. An insulin response element (IRE) (Suwanickul, 1993) is present at position -575 to -570. A CACCC box (Dierks *et al.*, 1983) is present at position -586 to -582 and a cyclic AMP response element (CRE) (Montminy and Bilezikjian, 1987) is present at position -687 to -683.

Northern Blot Analysis

Northern blot analyses of several tissues at E15 reveal a single transcript of approximately 2.3 kb (Fig. 18). The IGFBP-2 mRNA appears to be relatively abundant in E-15 eye, brain, skeletal muscle, heart and intestine. In contrast, E-15 liver exhibits a very low level of expression. For comparative purposes, human retina RNA was included in the northern analysis. As has been previously shown (Agarwal *et al.* 1991), the size of the human retinal IGFBP-2 transcript is approximately 1.5 kb, 800 bp smaller than the avian IGFBP-2 transcript.

Discussion

As was the case for the bovine cDNA (Upton *et al.*, 1990), IGFBP-2 clones isolated from the avian E-18 retina cDNA library were truncated at their 5′ end. In order to obtain the complete cDNA sequence it was necessary to obtain both 5′ RACE and genomic sequence. The inability to obtain the complete 5′ end from the RACE technique alone, was most likely due to the presence of significant secondary structure in exon-1, since this region was found to be extremely GC+ rich

(85%). Analysis of the avian IGFBP-2 sequence using an RNA secondary structure analysis program (Zuker and Stiegler, 1981) revealed numerous potential hairpins which could have interfered with reverse transcription and extension reactions.

The avian IGFBP-2 transcript of approximately 2.3 kb, is significantly larger than its counterparts in the rat, sheep, cow and human, all of which are around 1.5 kb. This size difference is the result of an unusually long (~1100 bp) 3' UTR in the chicken IGFBP-2 cDNA. Several studies have shown that the presence of multiple ATTTA motifs coupled with an AT-rich composition of the 3' UTR, may lead to mRNA instability (Shaw and Kamen, 1986, Ohme-Takagi *et al.*,1993). However, the presence of only one ATTTA motif in the 1100 bp 3' UTR along with the fact that the avian 3' UTR is not AT rich (47% AT), suggests that the avian IGFBP-2 mRNA may not necessarily be unstable. Future deletion studies of the 3' UTR should help to clarify its role in mRNA stability.

The deduced amino acid sequence of the avian IGFBP-2 cDNA is similar to those of several mammalian species including the rat (71%), sheep (68%), cow (68%) and human (68%) (Brown *et al.*1989; Upton *et al.*1990; Delhanty and Han, 1992, Binkert *et al.*1989). The positions of the 18 cysteine residues, that are critical for the binding of ligand and are conserved across the various mammalian IGFBPs, are also conserved in the avian IGFBP-2.

Optimal alignment of the amino acid sequence from several species reveals that the amino terminus of the human IGFBP-2 contains a 14 amino acid proline- rich stretch that is not

present in the chicken or the rat (Fig. 13., amino acids 18-32). The finding that both the chicken and the rat IGFBP-2 protein are missing these 14 amino acids raises the possibility that an insertion/duplication event may have occurred in this region of the gene, sometime during the divergence of the mammals. This idea is supported by the finding that only 4 amino acids are missing for the cow and sheep IGFBP-2. Although unlikely, the absence of the 14 amino acids in the chicken and rat could be the result of a deletion process such as replication slippage, which occurs during meiosis in regions of short tandem repeats (Inglehearn *et al.*, 1991).

The RGD (Arg-Gly-Asp) motif, present in mammalian IGFBP-2, is also retained in the avian IGFBP-2. The RGD peptide is involved in cell surface interactions with integrins (Hynes, 1987), and may be important for IGFBP interactions with target cells. In support of this idea, recent studies in which the RGD sequence of IGFBP-1 was mutated resulted in a decreased ability of IGFBP-1 to stimulate cell migration (Jones *et al.*1993). Unlike the RGD motif, the putative ATP binding domain, present in the human, bovine and ovine IGFBP-2 cDNAs, is absent in both the chicken and the rat. This finding raises the question of the significance of this domain in the biological function of IGFBP-2.

As observed for the rat (Brown and Rechler, 1990), mouse (Landwehr *et al.*, 1993) and human (Ehrenborg *et al.*, 1991) IGFBP-2 genes, the chicken IGFBP-2 gene is present as a single copy consisting of 4 exons and 3 introns that spans

approximately 38 kb. These similar features indicate that the gene has been conserved through its evolutionary development. Both the size of the exons and introns as well as the position of the splice junctions are conserved between mammals and birds as exemplified by the chicken. Interestingly, the large size of intron 1, which is ~32 kb in the chicken, 27 kb in the mouse and 28 kb in human, is also relatively well conserved in size. Although the size of intron 1 in the rat is not known, it is estimated as being "larger than 2.3 kb" (Brown and Rechler, 1990).

The 5′ flanking region of the chicken IGFBP-2 gene, like the mammalian IGFBP-2 gene, is TATA-less and CAAT-less. A common finding of similar TATA-less and CAAT-less genes is the interaction of GC rich regions with positive trans-acting transcription factors, such as Sp-1 (Kadonga *et al.*, 1986). Recent studies of the rat IGFBP-2 promoter region demonstrate that three Sp-1 sites are required for efficient transcription (Boisclair *et al.*, 1993). In addition to three Sp-1 sites, the putative promoter region of the chicken IGFBP-2 gene contains a pan element also present in the insulin gene (Nelson *et al.*, 1990) and in the rat IGFBP-2 gene. Furthermore, insulin (IRE) and cyclic AMP (CRE) response elements that are present in the human IGFBP-1 gene (Suwanickul *et al.*, 1993) are also present in the 5′ upstream region of the chicken IGFBP-2 gene. The finding of cAMP and insulin response elements in particular suggests an important role for these regulators in IGFBP-2 gene expression during chicken ocular development.

Previous studies have shown that the IGFBP-2 gene is, in fact, expressed at high levels early in development (Ooi *et al.* 1990; Orlowski *et al.* 1990). In agreement, with these studies, the avian IGFBP-2 is abundantly expressed in several E15 tissues. The high level of IGFBP-2 expression in a variety of different embryonic tissues suggests that IGFBP-2 is being utilized locally during mid-embryogenesis. The finding of a low expression of IGFBP-2 in the embryonic liver parallels a similar trend that has been found for mRNA levels in developing monkey (Liu *et al.* 1991) and sheep (Delhanty and Han, 1992) liver. It is logical to assume that the low expression of IGFBP-2 by E-15 liver is related to the concomitantly low level of serum IGFBP-2 (Yang *et al.* 1993),and raises the possibility that the IGFBP-2 in the serum at this stage of development may be derived from non-hepatic sources such as the yolk sac.

In summary, accumulating evidence indicates that IGFBP-2 may play an important role in vertebrate development and functioning in the mature animal as well. The finding of a high degree of conservation for IGFBP-2 between mammals and a non-mammalian species also supports the concept that IGFBP-2 is an important protein. The acquisition of the avian IGFBP-2 sequence has enabled us to study the expression and cellular localization of the IGFBP-2 transcript during the well-documented course of chicken development. In addition, further functional analysis of the promoter region of the chicken IGFBP-2 gene should prove useful in elucidating the regulation of tissue-specific expression.

Fig. 11. **Use of primers from a conserved region of the mammalian IGFBP-2, to obtain a 154 bp, chicken-specific, IGFBP-2 PCR product.** A. Human, bovine and rat IGFBP-2 amino acid sequences from a conserved region of exons 3 and 4 were aligned and primers (bold) constructed from the corresponding nucleic acid sequence. B. A single product of 154 bp was obtained using PCR on cDNA from several embryonic day 18 ocular tissues as well as liver. Products were electrophoresed on a 6% TBE gel and stained with ethidium bromide as described in the *Materials and Methods*.

A

```
        201                       225
Human   RISTMRLPDERGPLEHLYSLHIPNCDKHGLYNLKQCKMSLNGQRGECWC
Bovine  RISTMRLPDDRGPLEHLYSLHIPNCDKHGLYNLKQCKMSLNGQRGECWC
Rat     RISTMRLPDDRGPLEHLYSLHIPNCDKHGLYNLKQCKMSLNGQRGECWC
```

B

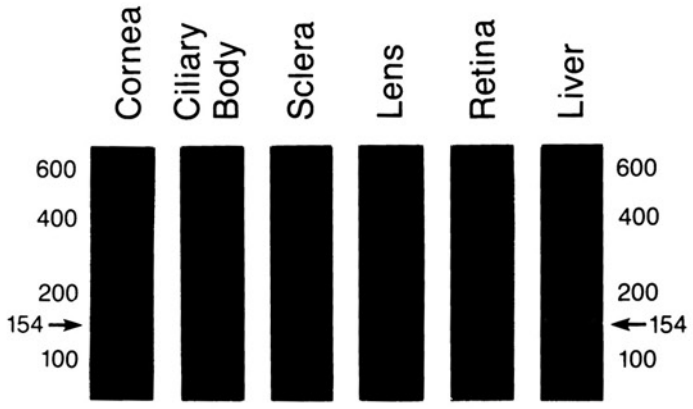


Fig. 12. **Nucleotide and deduced amino acid sequences of the chicken IGFBP-2 cDNA.** Sequence obtained from the 5′ RACE technique is singly underlined, while sequence obtained from genomic DNA is doubly underlined. The CCGCC Kozak consensus sequence, ATTTA instability motif and AATAAA polyadenylation signals are boxed, while the RGD motif is boxed and shaded. The Genbank accession number is gb:U15086.

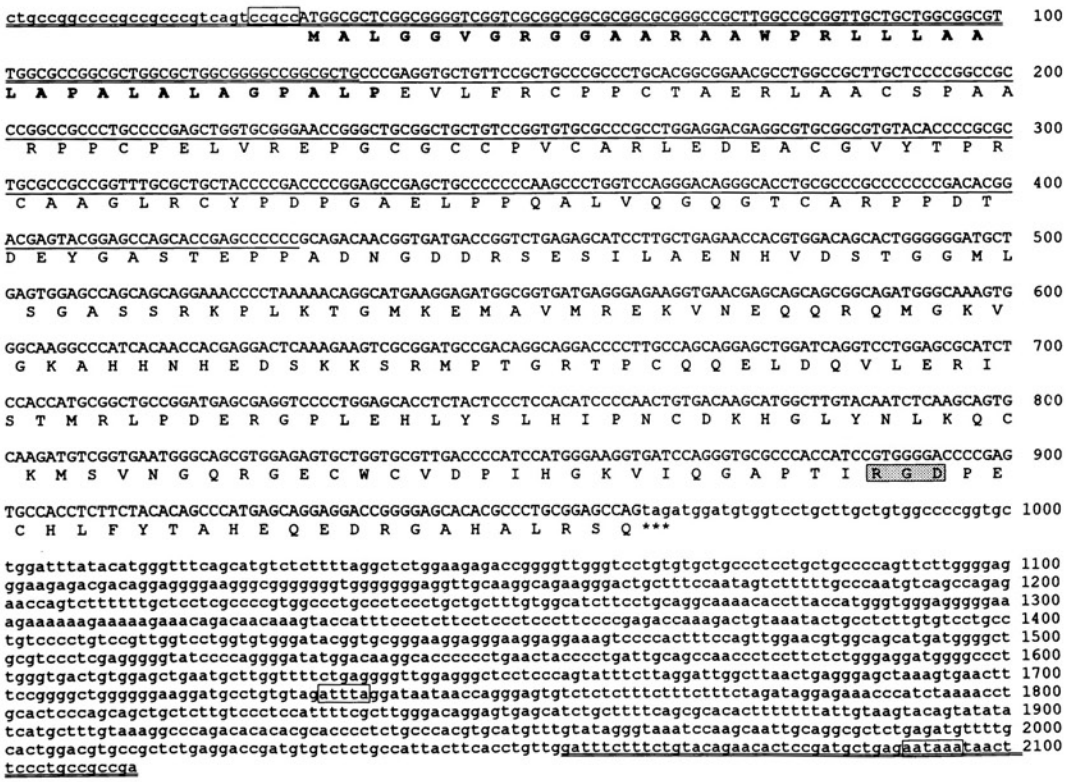


Fig. 13. **Comparison of the deduced peptide sequences of chicken, rat, cow, sheep, and human IGFBP-2.** The numbering of amino acids is based on the human sequence beginning with the first residue of the mature protein. Missing residues are indicated by dashes. Total numbers of amino acids and percentages of identity to the chicken IGFBP-2 are shown at the end of the sequence. The 18 conserved cysteine residues are shaded.

Fig. 14. **Southern blot analysis of the chicken IGFBP-2 gene.** Chicken genomic (10 μg/lane) and Cosmid 9A-1 DNA (300 ng/lane) was digested to completion with EcoRI, BamH-I and HindIII. The blot was hybridized with a PCR probe encompassing exon 2. Relative positions of hybridizing bands are indicated in kilobases.

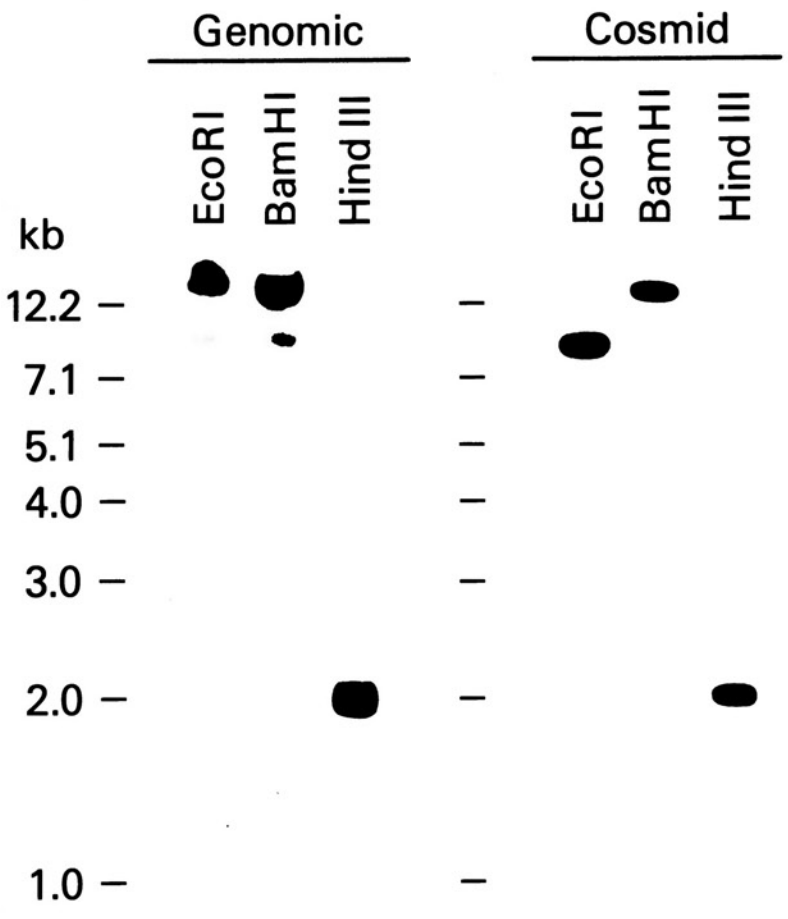

Genomic
Cosmid
EcoRI
BamHI
Hind III
EcoRI
BamHI
Hind III
kb
12.2
7.1
5.1
4.0
3.0
2.0
1.0

Fig. 15. **Restriction map of the chicken IGFBP-2 gene.** Exons are indicated by solid boxes. Restriction sites identified are: B, BamHI, E, EcoRI, K, KpnI. The distance between exons is measured in kb and is drawn to scale. Areas that are not drawn to scale are indicated by dotted lines. Only the edges of intron 1 are shown. Corresponding regions of the IGFBP-2 gene contained in cosmid clones 1A, 9A-1 and 2A-1 are shown above the map. Cosmid clone sequence that extends outside the chicken IGFBP-2 gene is indicated by jagged lines.

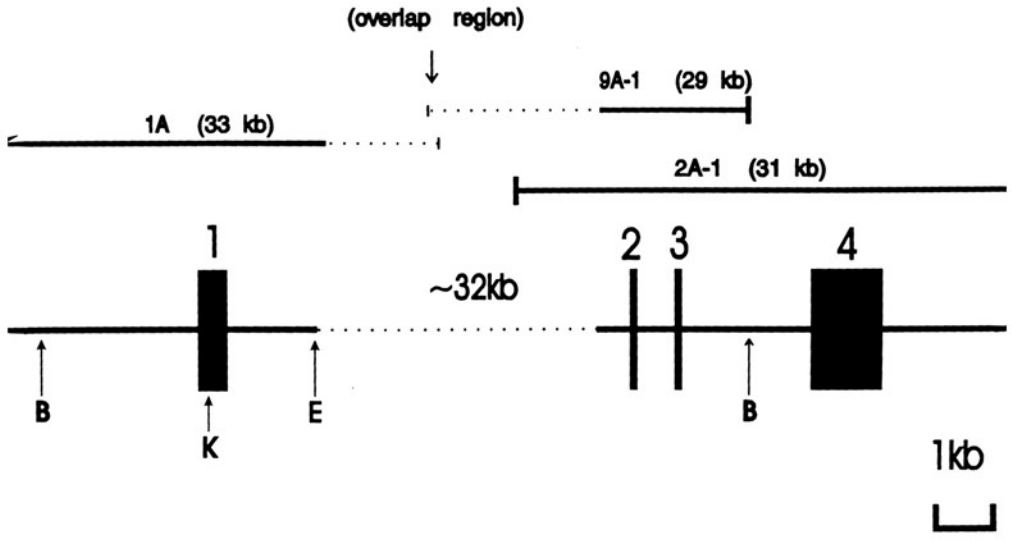

(overlap region)
9A-1 (29 kb)
1A (33 kb)
2A-1 (31 kb)
1
2 3
4
~32kb
B
K
E
B
1kb

Fig. 16. **Exon/Intron boundaries of the chicken IGFBP-2 gene.** Exons 1-4 and exon-intron boundaries were sequenced as described in Materials and Methods. Nucleotide sequence from the exons are capitalized, while intron sequences are shown in lower case letters. Dotted lines represent continuing intron sequence. Deduced amino acid sequences are shown above exon sequence as three letter abbreviations. Amino acid position in the avian IGFBP-2 gene is indicated by superscript numbers.

Exon	Exon size (bp)	5′ Splice Donor	3′ Splice Acceptor	Intron size (kbp)
1	~550	Pro Pro Ala[133] CCC CCC GCA G**gt**gagcag.....tttcctgc**ag**AC AAC GGT	Asp Asn Gly	~32
2	225	Thr Gly Arg[209] ACA GGC AGG**gt**gagaggag.....ttttttc**ag**ACC CCT TGC	Thr Pro Cys	0.7
3	141	Leu Lys Gln[256] CTC AAG CAG**gt**agtagaga.....tctcaagc**ag**TGC AAG ATG	Cys Lys Met	~1.9
4	~1300			

Fig. 17. **5′ Flanking region of the avian IGFBP-2 gene.** The sequence of the avian IGFBP-2 gene from the translation start site (ATG +1) to -661 is shown. Putative promoter elements such as the cyclic AMP element (CRE), CACC box, insulin response element (IRE), Sp1 site, and pan motif are boxed.

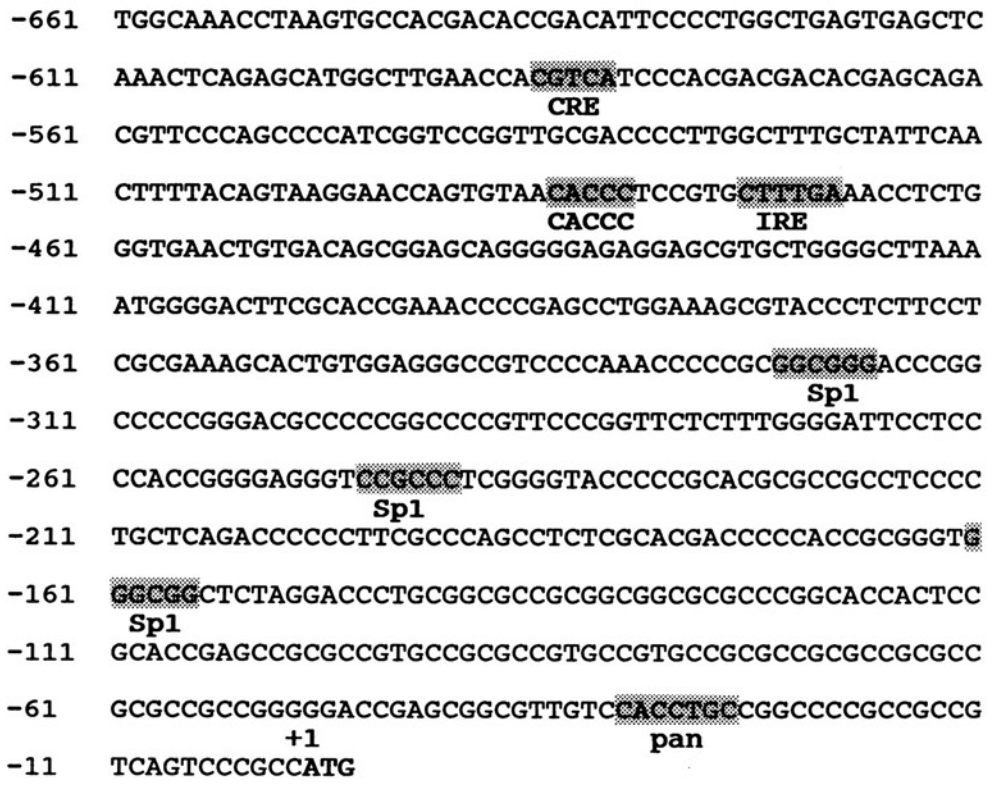
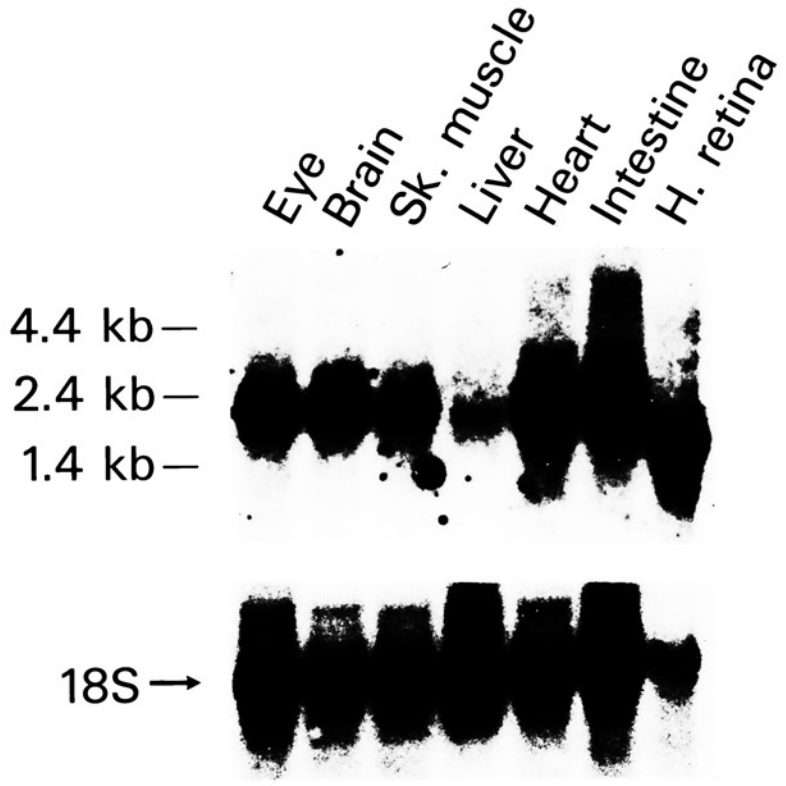

-661 TGGCAAACCTAAGTGCCACGACACCGACATTCCCCTGGCTGAGTGAGCTC
-611 AAACTCAGAGCATGGCTTGAACCACGTCATCCCACGACGACACGAGCAGA
CRE
-561 CGTTCCCAGCCCCATCGGTCCGGTTGCGACCCCTTGGCTTTGCTATTCAA
-511 CTTTTACAGTAAGGAACCAGTGTAACACCCTCCGTGCTTTGAAACCTCTG
CACCC
IRE
-461 GGTGAACTGTGACAGCGGAGCAGGGGGAGAGGAGCGTGCTGGGGCTTAAA
-411 ATGGGGACTTCGCACCGAAACCCCGAGCCTGGAAAGCGTACCCTCTTCCT
-361 CGCGAAAGCACTGTGGAGGGCCGTCCCCAAACCCCCGCCGCGGGACCCGG
Sp1
-311 CCCCCGGGACGCCCCCGGCCCCGTTCCCGGTTCTCTTTGGGGATTCCTCC
-261 CCACCGGGGAGGGTCCGCCCTCGGGGTACCCCCGCACGCGCCGCCTCCCC
Sp1
-211 TGCTCAGACCCCCCTTCGCCCAGCCTCTCGCACGACCCCCACCGCGGGTG
-161 GGCGGCTCTAGGACCCTGCGGCGCCGCGGCGGCGCGCCCGGCACCACTCC
Sp1
-111 GCACCGAGCCGCGCCGTGCCGCGCCGTGCCGTGCCGCGCCGCGCCGCGCC
-61 GCGCCGCCGGGGGACCGAGCGGCGTTGTCCACCTGCCGGCCCCGCCGCCG
+1
pan
-11 TCAGTCCCGCCATG

Fig. 18. **The expression of IGFBP-2 mRNA in embryonic day 15 chicken tissues and in adult human retina.** Total RNA (5 μg/lane) was electrophoresed, blotted and probed with ^{32}P-labeled 154 bp chicken PCR product. To normalize loading and transfer variations between lanes, the blot was stripped and reprobed with a chicken 18S rRNA probe.


Eye
Brain
Sk. muscle
Liver
Heart
Intestine
H. retina
4.4 kb
2.4 kb
1.4 kb
18S

CHAPTER V

Differential Expression and *In-situ* Localization of Insulin-Like Growth Factor Binding Protein-2 in Developing Chicken Ocular Tissues

Introduction

In order to identify the specific ocular cell types containing IGFBP-2 mRNA, as well as to study the temporal and spatial distribution of IGFBP-2 expression in developing ocular tissues, *in situ* hybridization was performed. An ^{35}S-riboprobe was generated from the chicken IGFBP-2 plasmid cDNA clone and used to analyze paraffin sections of formaldehyde fixed eyes at E6 and E12. In order to get an estimation of the relative levels of IGFBP-2 mRNA in ocular tissues, northern blot analyses were performed on cornea, retina and sclera from embryonic days 8, 12, 18 and 2 days post-hatching.

Results

In Situ Hybridization

By embryonic day 6 (E6), the cornea consists of an epithelium, narrow stroma and endothelium (Fig. 19A,C,E). At this stage, IGFBP-2 mRNA is present in the developing epithelium and endothelium but is absent from the stroma (Fig.

19C). However, by E12, the corneal epithelium, stroma and endothelium are almost fully developed (Fig. 19B), and IGFBP-2 expression is observed in all three layers as well as in other ocular structures such as the eyelid and ciliary body (Fig. 19D). E6 and E12 sections probed with the sense strand show only weak background reactivity (Fig. 19E, F).

At E6, the neural retina (NR) contains a single lamina of undifferentiated neuronal cells (Fig. 20A,C,E). At this stage of development, IGFBP-2 mRNA is diffusely distributed across the neural retina (Fig. 20C). A higher level of IGFBP-2 mRNA expression is observed in the E6 sclera (Fig. 20C). However, by E12, the retina has differentiated into distinct ganglion cell, inner plexiform, inner nuclear, outer plexiform and outer nuclear layers (Fig. 20B), although photoreceptor outer segments have yet to form. At this stage, the expression of IGFBP-2 mRNA is generally confined to layers of the retina containing the cell bodies, eg. ganglion cell, inner nuclear and outer nuclear layers and, as might be expected, is absent from the plexiform layers (Fig. 20D). Control sections of both E6 and E12 retina probed with the sense strand show only low background labeling (Fig. 20, E,F). Little or no specific labeling was observed in lens under these conditions (data not shown).

Fig. 21 focuses on the histological appearance of the retina-pimented epithelium (RPE)-choroid-sclera complex at E12, demonstrating that it is relatively well-formed and delineated at this stage of development. Fig. 22B shows that IGFBP-2 mRNA is diffusely expressed across the entire RPE-

choroid-sclera complex at this time. Control sense sections are only weakly labeled (Fig. 21C).

Northern Blots

In order to compare the relative abundance of mRNA in cornea, retina and sclera, northern blots were performed on each of the tissues from different stages of development (Fig. 22A). IGFBP-2 autoradiographs were normalized to the relative intensities of 28S and 18S bands stained with ethidium bromide (Fig. 24B). The results of assays from three separate groups of animals are graphically represented in Fig. 24C.

Of the stages examined, IGFBP-2 mRNA expression in the cornea is highest at E8 (Fig. 22, left panel). By E12, IGFBP-2 mRNA expression decreases by approximately 50% and then remains relatively low from E18 through hatching (P-2). In contrast to the cornea, expression of IGFBP-2 mRNA in the retina (Fig. 22, middle panel) is low at embryonic day 8. This increases approximately two-fold by E12 and peaks at E18. After hatching (P-2), IGFBP-2 mRNA expression decreases to approximately 60% of that observed at E18. The developing sclera exhibits an IGFBP-2 expression pattern similar to that observed for the cornea. The highest level of IGFBP-2 mRNA expression is observed at E8, i.e., early in ocular development (Fig. 22, right column). Thereafter, IGFBP-2 mRNA expression decreases somewhat and, at P-2, expression decreases to about 50% of that observed at E8.

In order to more accurately compare the relative levels of IGFBP-2 message in the different ocular tissues with each

other as well as with other non-ocular tissues, northern blot analyses were performed on P-2 cornea, retina, sclera and, for comparison, brain and liver (Fig. 23A). Blots were normalized using an 18S ribosomal probe (Fig. 23B). A graphic representation of the results shown in Fig. 23C.

As expected, both liver and brain tissues from P-2 animals exhibit a high level of IGFBP-2 expression. Surprisingly, however, the cornea exhibits a level of IGFBP-2 expression that is equivalent to that observed in the two reference tissues. The P-2 retina and sclera exhibit approximately 60% of the IGFBP-2 expression observed in brain. In contrast, the lens (fibers + epithelium) exhibits only a very weak signal for IGFBP-2 mRNA.

Discussion

The results of the present study demonstrate that, as early as six days of development, IGFBP-2 mRNA is already expressed in a number of ocular tissues including the cornea, retina and sclera. By embryonic day 6, migrating neural crest cells, destined to form head mesenchyme, have invaded the space between the corneal epithelium and lens and have begun to form the corneal stroma and endothelium (Johnston *et al.*, 1979). At this time, IGFBP-2 mRNA is not only observed in the corneal epithelium but in the endothelium as well as surrounding structures such as the eyelid and ciliary body. The high level of IGFBP-2 expression in the corneal epithelium during the formation of the stroma raises the possibility that

IGFBP-2 may be involved in regulating the migration and differentiation of invading mesenchymal cells. In support of this idea, IGFBP-1 is able to stimulate fibroblast migration via its interaction with α_5 β_1 integrin (Jones *et al.*, 1993) and IGFBP-2, like IGFBP-1, contains the RGD peptide that has shown to be important for cell surface binding to integrins (Hynes, 1987). A similar pattern of expression is also observed in limb development where IGFBP-2 mRNA is concentrated in the apical ectodermal ridge and IGF-I and -II mRNAs are found in the adjacent mesoderm (Streck *et al.*, 1992). Although the localization of IGF mRNA was not investigated in the present study, IGF-II mRNA is highly expressed by mesenchymal tissues forming the sclera and corneal stroma (Cuthbertson *et al.*, 1989). Importantly, we have previously shown that IGFBP-2 binds IGF-II as well as IGF-I (Arnold *et al.*, 1993).

As judged by both *in situ* hybridization and northern blot analysis, the level of IGFBP-2 mRNA expression in several important ocular tissues correlates well with their state of mitotic activity and differentiation. Early in development (E8), there is a high level of IGFBP-2 mRNA expression in both the cornea and sclera at a time when these tissues are rapidly proliferating (Nuttal, 1976; Van De Kamp and Hilfer, 1985). The presence of high levels of IGFBP-2 in the cornea and sclera during this early growth phase of the eye suggests a role for IGFBP-2 in facilitating the proliferative effects of IGF on these tissues. In support of this idea, previous studies have shown that IGFBP-2 functions

synergistically with IGF-I to promote smooth muscle cell proliferation *in vivo* (Bourner, et al, 1992). IGFBP-2 itself could be involved in this phase of ocular growth since mounting evidence indicates that it and other IGFBPs have intrinsic biological activity independent of their interactions with the IGFs (Booth *et al.*, 1991; Jones *et al.*, 1993).

In contrast to the cornea and sclera, IGFBP-2 expression in the undifferentiated E8 retina is low and increases as neuronal differentiation progresses. Expression peaks around E18, a time when cell proliferation has ceased in the retina, but when photoreceptor cells are undergoing rapid structural differentiation, eg. outer segment elongation (Coulombre, 1955). The high level of IGFBP-2 expression at this stage of development thus suggests a role for IGFBP-2 in regulating IGF-mediated neuronal maturation and differentiation, rather than in neuronal cell proliferation. In support of this idea, IGF-I has been shown to be important for the differentiation and survival of other neuronal cell types *in vitro* (Svrzic and Schubert, 1990). In addition, IGF-I and insulin in combination with βFGF stimulate the *in vitro* differentiation of retinal precursor cells into a rod photoreceptor-specific phenotype (Mack and Fernald, 1993). Our additional finding that IGFBP-2 mRNA expression is mainly confined to the nuclear layers of the retina, and is not observed in the plexiform layers supports the concept that IGFBP-2 may be synthesized and released locally by the neural cell soma, as opposed to transport and dendritic release (Lee *et al.*, 1992).

Northern blot comparisons of ocular tissues with brain and liver at P-2 reveal that, among the ocular tissues examined, cornea has the highest IGFBP-2 expression, although IGFBP-2 expression is maintained in the sclera and retina at relatively high levels. Previous IGF binding studies have demonstrated that the cornea has the highest level of IGFBPs among ocular tissues and fluids (Arnold *et al.*, 1993). The finding of a high level of both IGFBP-2 mRNA and protein in the cornea, coupled with evidence that both IGF-I (Hyldahl, 1986) and IGF-II (Storckenfeldt *et al.*, 1991) are able to stimulate the proliferation of several corneal cell types, strongly suggests that IGFBP-2 is involved in the growth of the cornea and the sclera as well. In this regard, it would be interesting to investigate a possible role for IGFBPs in myopia and other structural diseases of the eye. High levels in the postnatal cornea and sclera also indicate a continuing role for IGFBPs in the maintenance of these mature tissues. Similarly, in the retina, maintenance of IGFBP-2 expression in the postnatal period, well after cessation of cellular proliferation and attainment of full differentiation indicates an important role of this IGFBP in mature retinal cell functioning as well.

In summary, the results of the present study reveal a differential expression of IGFBP-2 mRNA in several tissues of the developing chicken eye. Although it is not yet clear if IGFBPs are involved in the modulation of IGF activity or have intrinsic biological activity in themselves, the unique temporal and spatial expression of IGFBP-2 in developing

ocular tissues suggests a role for this BP in the regulation of ocular growth and differentiation.

Fig. 19 **H & E bright field sections of (A) cornea at embryonic day 6 and (D) embryonic day 12 anterior segment.** Darkfield illumination reveals IGFBP-2 mRNA in the epithelium and endothelium of the E6 cornea (B). It is uniformly distributed across the eyelid (lid), cornea (COR) and ciliary body (C.B.) of the E12 anterior segment (E). No labeling is observed in control sections probed with the sense strand in E6 cornea (C) or E12 anterior segment (F). A.C.= anterior chamber. Scale bar = 25 μm (A,B,C), 100 μm (D,E,F).

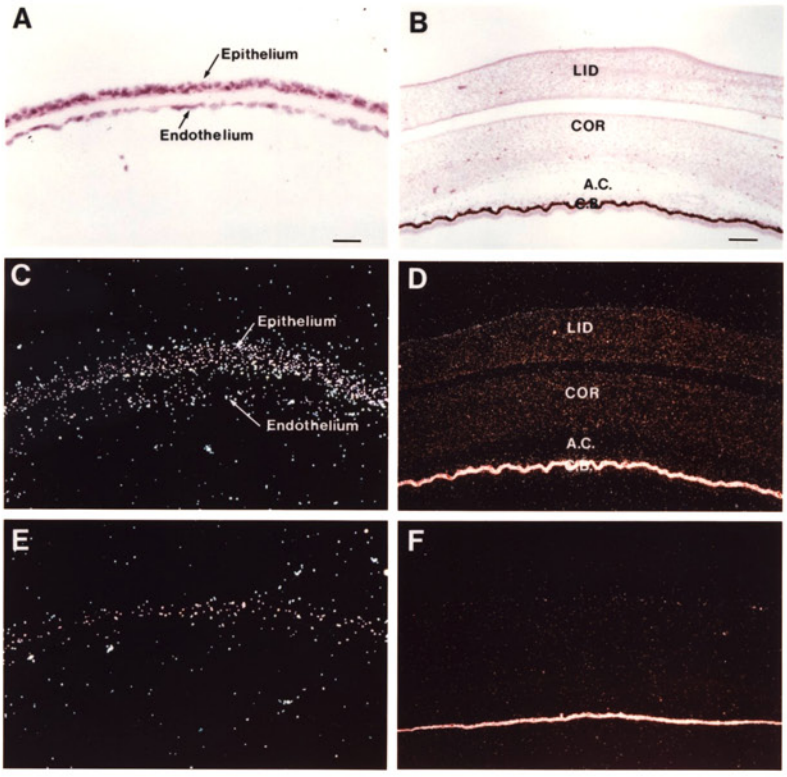

A
Epithelium
Endothelium
B
LID
COR
A.C.
C
Epithelium
Endothelium
D
LID
COR
A.C.
E
F

Fig. 20. **H & E brightfield sections of embryonic day 6 (E6) (A) and embryonic day 12 (E12) (B) retina.** Darkfield illumination shows that IGFBP-2 mRNA expression is diffusely distributed across the E6 neural retina (NR) (C) and is also expressed in the sclera (SCL). Intense reactivity of the retinal pigmented epithelium (RPE) is caused by light scattering melanin granules and is not due to IGFBP-2 mRNA as it is also observed in the control sense sections (E). By E12 (D), IGFBP-2 mRNA expression is confined mostly to the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL). Little or no labeling is observed in the inner plexiform layers (IPL) and in control sense section (F). Scale bar = 25 μm

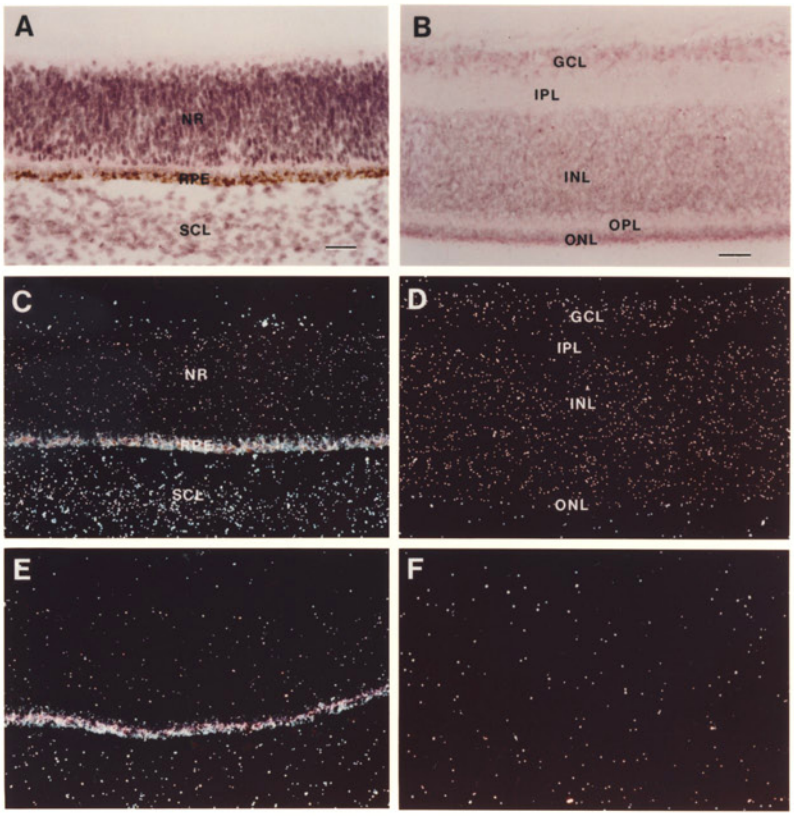

A
NR
RPE
SCL
B
GCL
IPL
INL
OPL
ONL
C
NR
RPE
SCL
D
GCL
IPL
INL
ONL
E
F

Fig. 21. **Bright field illumination of an H & E-stained section through an embryonic day 12 posterior eye cup (A) showing the retinal pigment epithelium (RPE), choroid (CH), cartilage (C), blood vessels (BV) and sclera (SCL).** Darkfield illumination shows a diffusely distributed expression of IGFBP-2 mRNA across the sclera (B). Intense reactivity of the retinal pigment epithelium (RPE) in (B) is mainly caused by light-scattering melanin granules as it is also observed in the control, sense-strand sections (C). Scale bar = 25 μm

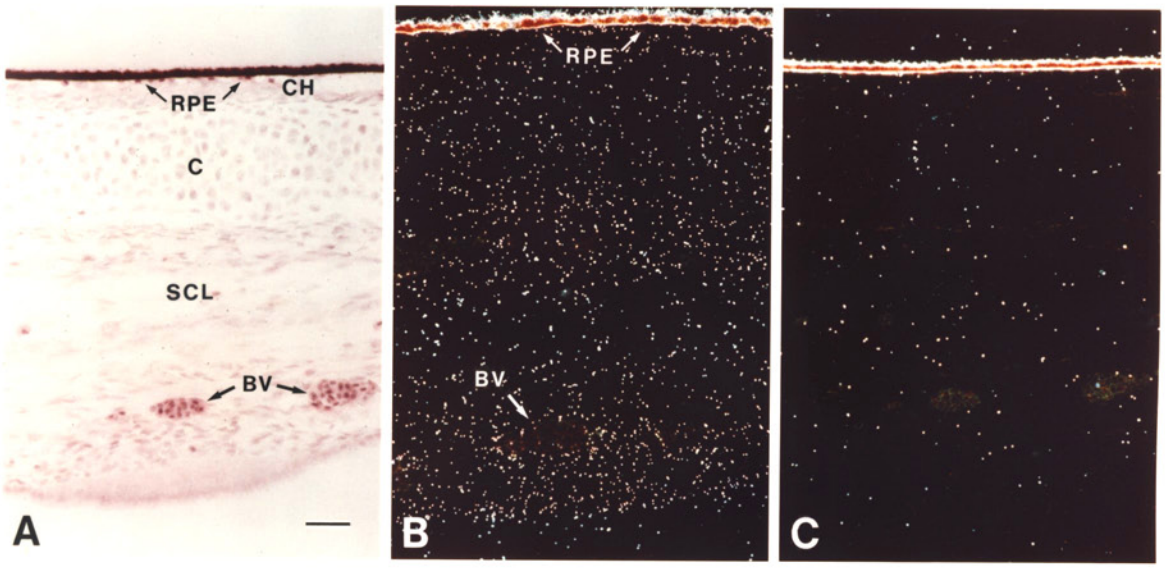

RPE
CH
C
SCL
BV
A
RPE
BV
B
C

Fig. 22. **Northern blot analysis of IGFBP-2 mRNA expression in developing ocular tissues.** Samples of total RNA (5 μg) from cornea, retina and sclera at embryonic day 8, 12, 18 and post-hatching day 2 were electrophoresed on a 1% agarose-formaldehyde gel, transferred to a nylon membrane and probed for IGFBP-2 (A). Ethidium bromide-staining (B) demonstrates RNA integrity and the relative lack of variation in loading of individual lanes. Autoradiographs were scanned and normalized to the relative intensity of the 28S and 18S bands as shown in the histograms (C). Results are expressed as the mean values ±SEM for three different sets of animals as described in *Materials and Methods*.

RELATIVE LEVELS OF IGFBP-2 mRNA DURING DEVELOPMENT

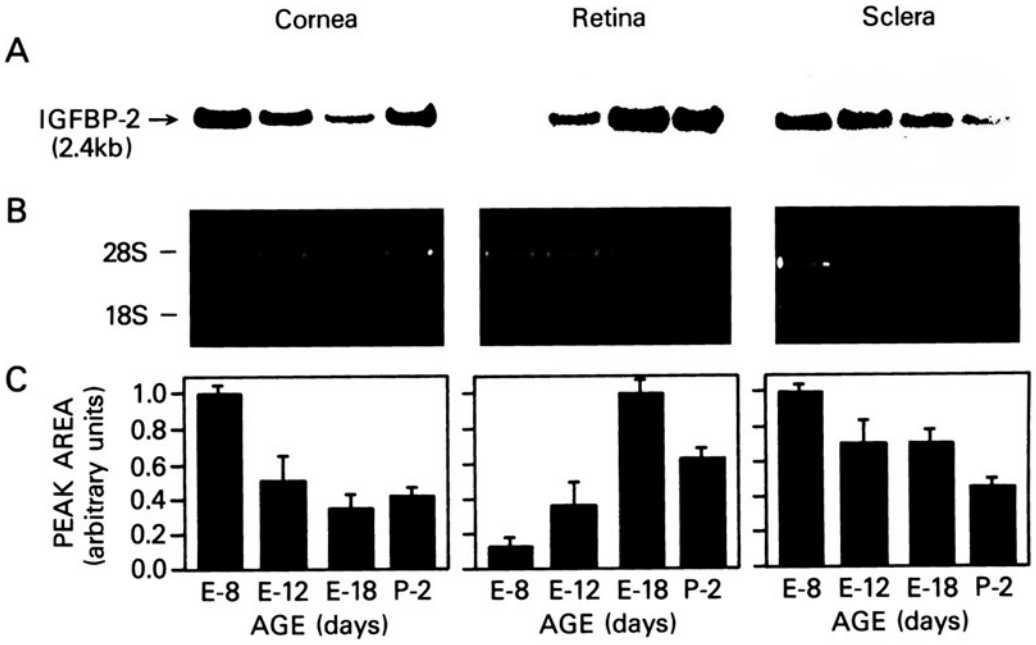
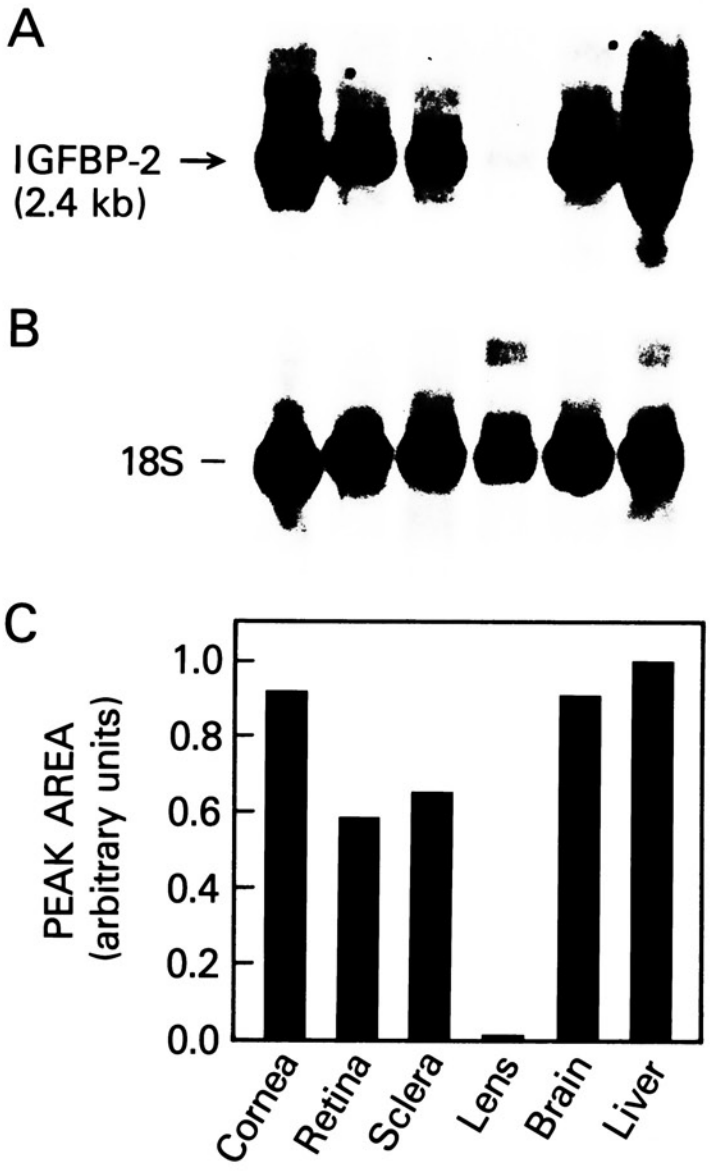


Fig. 23. **Northern blot analysis of IGFBP-2 mRNA expression in post-hatching day 2 tissues.** Five μg total RNA from cornea, retina, sclera, lens, brain and liver was electrophoresed on a 1% agarose formaldehyde gel, transferred to a nylon membranes and probed for IGFBP-2 (A). Individual loading variation was normalized by re-probing the blot with an 18S ribosomal probe (B). A graphic representation of the results is shown in C.


A
IGFBP-2 →
(2.4 kb)
B
18S –
C
PEAK AREA
(arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
Cornea
Retina
Sclera
Lens
Brain
Liver

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

The present study reveals three major findings. First, IGF competitive binding, western ligand blotting and affinity crosslinking studies demonstrate the presence of several specific, high affinity IGFBPs in embryonic and post-hatching vitreous humor. Vitreal IGFBPs exhibit a unique pattern of developmental expression as compared to serum IGFBPs, suggesting that they may be synthesized and regulated locally by ocular tissues. Secondly, analysis of the cDNA and gene for IGFBP-2, the major IGFBP in ocular tissues and fluids, demonstrates a high degree of sequence conservation as compared to mammalian IGFBP-2. Finally, northern blot and *in situ* hybridization studies utilizing chicken-specific IGFBP-2 probes, show a distinct spatial and temporal developmental expression of IGFBP-2 mRNA in the cornea, retina and sclera, implying tissue-specific functions.

The findings of the present study raise the question of whether IGFBP-2 is of importance in the regulation of ocular growth and development. Following are a number of potentially useful directions that might better establish the role of IGFBP-2 in ocular tissues:

1) ARE OCULAR TISSUES AFFECTED BY IGFBP-2 GENE ABLATION?

Clearly, the most direct way to address this question, is to block the expression of IGFBP-2 in the developing embryo.

Surprisingly, transgenic mice in which the IGFBP-2 gene was "knocked out" by homologous recombination, revealed no "gross" abnormalities in general development (Wood *et al.*, 1993). Since several other IGFBPs (IGFBP-4, 5, and -6) are present in multiple tissues of the IGFBP-2 knockout mouse, the possibility that they may act to compensate for the absence of IGFBP-2 must be considered. Alternatively, ocular abnormalities may be present in the IGFBP-2 knockout mouse, since a careful histological analysis of ocular tissue was not conducted (personal communication from Dr. John Pintar). A collaborative effort is currently underway to determine if ocular tissues from the IGFBP-2 knockout mice are, in fact, histologically and biochemically normal.

2) IS IGFBP-2 INVOLVED IN THE REGULATION OF CELL PROLIFERATION IN THE EMBRYONIC CORNEA?

The cornea is composed of three cellular layers: an outer epithelium, an intermediate stroma and an inner endothelium. The epithelium is derived from surface ectoderm that is induced by interactions with the lens vesicle and optic cup (reviewed by Coulombre, 1965). In contrast, the stromal cells or keratocytes and the inner endothelium, are derived from neural crest cells (Johnston *et al.*, 1979). The present northern blot studies show that the cornea expresses a high level of IGFBP-2 mRNA early in development (E-8 through E-12). In addition, *in situ* hybridization reveals that IGFBP-2 mRNA is present as early as 3.5 days of embryonic development in the presumptive corneal epithelium (Schoen *et al.*, 1995). A

high level of IGFBP-2 mRNA expression in the corneal epithelium is also observed at embryonic day 6 when epithelial and stromal cells are undergoing rapid proliferation. This finding suggests a role for IGFBP-2 in this process. Interestingly, IGFBP-2 has been shown to both enhance and inhibit the mitogenic effects of IGF-I *in vitro,* depending on the cell type. For example, IGFBP-2 augments IGF-I-stimulated incorporation of ^{3}H-thymidine in smooth muscle cells (Bourner *et al.*, 1992) and breast carcinoma cells (Chen *et al.*, 1994), whereas it suppresses the binding of radiolabeled IGF-I and -II to small cell lung carcinoma cells and inhibits IGF-I-stimulated DNA synthesis by these cells (Reeve *et al.*, 1993).

In order to study the function of IGFBP-2 in the proliferation of embryonic corneal epithelium and stromal cells, an organ culture system would be best utilized. Organ culture has several advantages over tissue culture and *in-vivo* manipulations. Primarily, in organ culture the integrity of the tissue is kept intact, with the maintenance of cell to cell contacts. This is in contrast to tissue culture, where individual cells are disassociated by trypsin and then replated. In addition, the environment surrounding the cultured tissue can be defined with respect to amounts of essential nutrients, growth factors and other constituents. This feature provides an advantage over the *in vivo* situation where the presence of exogenous growth factors and IGFBPs could complicate the experimental findings. However, organ culture is limited to short term (24 hr) studies and because the environment is somewhat different from the *in vivo*

environment, it is possible that the cells may respond differently than in the *in-vivo* situation. In addition, IGFs and IGFBPs may be produced locally by the cultured tissue, creating the need to study a range of different effective concentrations in order to "out compete" locally produced factors. Nevertheless, an organ culture system appears to be a useful means of investigating the role of IGFBP-2 in corneal development.

The developing cornea provides an ideal system for investigating the role of IGF-I and IGFBPs on cell proliferation and morphogenesis. Corneal development has been well documented (Coulombre, 1965) and a technique for temporary corneal organ culture has been established (Hyldahl, 1986).

To address the question of whether IGFBP-2 has a direct effect on corneal cell proliferation that is independent of IGF, corneas could be incubated in the presence of varying concentrations of IGFBP-2 (0-50 nM). Cell proliferation could be assessed using ^{3}H-thymidine incorporation and autoradiography. Using this technique, the number of labeled cells versus total number of cells (synthetic index) could be evaluated. Since endogenous IGF-I or IGF-II may be produced by the cultured ocular tissues, a function blocking antibody against IGF-I and -II could be added to the media as appropriate.

To investigate the potential positive or negative interactions of IGFBP-2 with IGF-I, corneas could be incubated in the presence of a range of different IGF-I concentrations

(0-50 nM) along with IGFBP-2 at concentrations ranging from 0 to 50 nM. In addition to IGF-I, Des 1-3, a truncated IGF-I variant that binds to the type I IGF receptor with normal affinity, but does not bind to IGFBPs, could also be tested. This should help clarify whether an interaction between IGFBP-2 and IGF-I is required for IGF-I to mediate an effect on cell proliferation.

IGFBP-2 contains the RGD peptide that is present in many proteins that interact with cell surface integrins. The potential interaction of IGFBP-2 with cell surface integrins may be a means for increasing the local concentration of IGF-I in proximity of its receptor and may lead to an increase in IGF activity. Therefore, it will be important to study the actions of the IGFBP-2 protein with and without a functional RGD motif. Previous studies have shown that mutating the RGD to WGD in IGFBP-1, is sufficient to abolish binding to cell surface integrin (Jones *et al.*, 1993). Accordingly, another set of experiments using a recombinant form of IGFBP-2 containing an RGD to WGD replacement could be also be performed. Alternatively, another approach to blocking IGFBP-2 from interacting with cell surface integrins, would be to add excess RGD peptide to the media. This would saturate all RGD-binding integrins and prevent IGFBP-2 from binding, but might also interfere with neuronal cell adhesion.

3) IS IGFBP-2 INVOLVED IN THE REGULATION OF NEURAL CREST CELL MIGRATION INTO THE CORNEAL STROMA?

Following detachment of the surface ectoderm from the

underlying lens vesicle at approximately stage 17 (embryonic day 2.5-3), the corneal epithelium begins secreting the primary stroma (Coulombre, 1965). Neural crest cells from the pericorneal region migrate between the anterior surface of the lens and the primary stroma to form a flattened, confluent epithelial cell layer known as the endothelium. After the formation of the endothelium, the primary stroma swells and neural crest cells begin to migrate from periocular mesenchyme and invade the stroma (Johnston *et al.*, 1979).

It is not clear what initiates the migration of neural crest cells into the corneal stroma. Studies have shown that neural crest cells will bind to specific cell adhesion molecules such as fibronectin and will migrate along a fibronectin-rich track away from regions of high cell density (Mayer *et al.*, 1981). The finding that neural crest cell migration can be altered by a variety of RGD containing proteins (Delannet *et al.*, 1994) raises the possibility that IGFBP-2 may be involved in neural crest cell migration into the corneal stroma. Evidence supporting a role for RGD-containing IGFBPs in the augmentation of cell migration, comes from studies in which Chinese Hamster Ovary (CHO) cells were transfected with IGFBP-1 containing a mutated RGD peptide. In these studies, a 3-fold decrease in the rate of migration of the CHO cells was found as compared to cells expressing the wild type IGFBP-1 (Jones, et. al., 1993). In this same study, $\alpha 5\beta 1$ integrin (fibronectin receptor) was identified as the cell surface protein interacting with IGFBP-1. IGFBP-2, like IGFBP-1, contains the RGD peptide that is known to interact

with cell surface integrins. Similar studies with IGFBP-2 also indicate an interaction with $\alpha 5\beta 1$ integrin (personal communication from Thomas Roszman).

To determine if IGFBP-2 is involved in facilitating the migration of neural crest cells into the corneal stroma, it will be necessary to first determine if IGFBP-2 is able to influence the migration of neural crest cells in a defined environment. Previous studies have shown that when a segment of neural tube containing neural crest cells from stage 9-10 embryos (29-38 hrs.) is placed in a petri dish containing a thin line of fibronectin plated on the substratum, neural crest cells will migrate along the fibronectin line in a non-random fashion (Rosavio *et al.*, 1983). Therefore, a similar experiment could be conducted using recombinant IGFBP-2. To evaluate the role of the RGD peptide in regulating neural crest migration, IGFBP-2 containing the mutated RGD peptide (WGD), could also be tested.

Assuming that the results of the above experiments indicate a role for IGFBP-2 in modulating neural crest cell migration, the next step will be to demonstrate that IGFBP-2 is involved in regulating the migration of neural crest cells *in vivo*. One way to determine if IGFBP-2 is involved in regulating neural crest cell migration into the corneal stroma is to eliminate IGFBP-2 in the cornea before neural crest migration has taken place and then observe if there is a decrease in the number of neural crest cells that migrate into the stroma. This observation may be made in the transgenic "IGFBP-2 knockout" mouse, which has not been specifically

examined for such abnormalities in the stroma. Antisense oligonucleotides offer another means of selectively disrupting the expression/translation of specific genes (Runyan *et al.*, 1992). Antisense IGFBP-2 oligonucleotides could be administered directly into the anterior chamber of the eye or into the amniotic cavity during early development, in an attempt to block the expression of IGFBP-2. Another approach would be to administer function-blocking antibodies against IGFBP-2 into the anterior chamber or amniotic cavity. Finally, excess RGD peptide could be administered to block IGFBP-2 from binding to integrins. However, this might also interfere with the binding of other RGD containing proteins, which could complicate the experimental interpretation.

Another means of investigating the role of IGFBP-2 in the cornea is to study the effect of supplementing endogenous IGFBP-2. A method for obtaining a sustained release of biologically active substances *in vivo* has been developed using ethlene/vinyl acetate copolymer (EVAc) (Langer and Folkman, 1976). Therefore, IGFBP-2 or a control protein such as albumin could be impregnated in an EVAc implant and placed in the corneal stroma. The number of neural crest cells migrating into the stroma containing the IGFBP-2 implant versus control protein could then be evaluated. Cell proliferation could also be assessed using ^{3}H-thymidine incorporation and autoradiography.

4) WHAT IS THE ROLE OF IGFBP-2 IN THE DEVELOPING SCLERA ?

Like the cornea, IGFBP-2 expression in the sclera is

highest early in development (embryonic day 6). The expression of IGF-I receptors in the sclera is also high early in development and decreases by approximately 50% between embryonic day 10 and the 2nd week after hatching (Waldbillig *et al.*, 1991). Likewise, the expression of IGF-II mRNA is also high in the loose mesenchymal tissue as it differentiates to form sclera, but not in the compact mature sclera (Cuthbertson *et al.*, 1989). Thus, the coordinate high expression of IGFBP-2, IGF-I receptor and IGF-II mRNA observed in the early stages of scleral development points to a role for the IGF system in scleral growth and differentiation. In order to determine if IGFBP-2 plays a role in scleral development, experiments similar to those described for studying the role IGFBP-2 in corneal cell proliferation and neural crest migration could also be conducted.

5) WHAT IS THE ROLE OF IGFBP-2 IN THE RETINA?

In contrast to the cornea and sclera, a fundamentally different temporal expression of IGFBP-2 is observed in the developing retina. Whereas IGFBP-2 expression is highest in the embryonic day 8 cornea and sclera, the retina shows a low level of IGFBP-2 mRNA expression at this stage. The finding that IGFBP-2 mRNA peaks in the retina at a developmental age (E-18) when cell proliferation has ceased, but when neuronal differentiation and synaptogenesis is taking place, suggests a role for IGFBP-2 in the latter process. In support of this idea, in the embryonic rat, IGFBP-2 is highly expressed in the floor plate area of the spinal cord, a region thought to be

important in regulating axonal guidance (Wood *et al.*, 1990). The finding that IGFBPs are restricted to the plexiform layers (regions of synaptic contacts) of the retina (Bassnett and Beebe, 1990; Waldbillig, unpublished observations), raise the possibility that IGFBP-2 may be involved in the terminal differentiation and synaptic maturation of retinal neurons.

Is IGFBP-2 involved in the terminal differentiation of retinal neurons? Because of the complexity of the neural retina and difficulty in assessing visual function, it would be a difficult task to assess the functional role of IGFBP-2 in this tissue *in vivo*. However, one approach would be to immunocytochemically assess the appearance of mature retinal markers (opsin, neuronal specific enolase, IRBP, NF-200, etc.) in retinas from control and "IGFBP-2 knockout" mice. Organ culture of chick retina has also been used to study the effects of various growth factors on differentiation (Tcheng *et al.*, 1994). Retinas at different stages could be incubated with combinations of IGFBP-2 and IGF-I and the effects monitored biochemically or immunocytochemically.

It may also be possible to gain some insight into whether or not IGFBP-2 is involved in neuronal differentiation by utilizing cultured retinoblastoma cells. Y-79 retinoblastoma cells are tumor cells derived from a primitive, multi-potential, retinoblasts and have been used as a model system for studying the effects of various agents on growth and differentiation (Kyritsis *et al.*, 1986). Although morphologically different than retinal neurons, retinoblastoma cells will at least partially differentiate into several

retinal neuronal phenotypes and express retina-specific proteins following treatment with specific differentiating agents. Importantly, it has already been shown that these cells will produce IGFBP-2 and that production is modulated by known differentiating agents. For example, treatment of Y-79 retinoblastoma cells with the differentiating agent HMBA (N, N, 1-hexamethylene-bis-acetamide) resulted in an 8-fold increase in the production of IGFBP-2 (Hayden *et al.*, 1992). Experiments are currently underway to determine if adding recombinant IGFBP-2 to retinoblastoma cells in culture will stimulate their differentiation.

6) IS IGFBP-2 INVOLVED IN THE DIFFERENTIATION OF LENS EPITHELIUM?

The lens is composed of two cell types: epithelial cells and fiber cells. The lens epithelium is present as a single cell layer that covers the anterior two thirds of the lens. At the equator, the epithelial cells elongate and differentiate into fiber cells, which make up most of the mass of the lens. Both lens epithelial and fiber cells have been shown to possess specific type I IGF receptors (Bassnett and Beebe, 1990). In addition, IGF-I and an IGF-I-like substance in vitreous humor are able to specifically stimulate lens epithelial cell elongation and differentiation (Beebe *et al.*, 1987; Beebe *et al.*, 1980). The lens does not appear to synthesize IGFBP-2, making it an ideal tissue to study the effects of exogenously-added IGFBP-2. Moreover, a model system for culture of the lens epithelium has been established

(Beebe *et al.*, 1987). Therefore, as described above, various combinations of IGF-I and IGFBP-2 could be added to lens epithelial explants and its effect on cell elongation determined.

The recent finding that IGF-I stimulates the expression of fibronectin receptors (Palmade *et al.*, 1994), raises the possibility of an interaction of these receptors with IGFBP-2. Therefore, it will be of interest to utilize the WGD-mutant form of IGFBP-2 in the above studies to determine if IGFBP-2 is interacting with fibronectin receptors.

In summary, the present dissertation has provided a fundamental framework of information regarding the sequence and developmental expression of IGFBP-2 in the chicken eye. This information should prove useful in the design of experiments, such as those described above, to help elucidate the role(s) of IGFBP-2 in ocular development. The abundance and early appearance of different IGFBPs in a wide variety of embryonic tissues indicates that these proteins probably play important roles in many diverse developmental processes. Due to the complexity of potential interactions between the assortment of IGFBPs, their ligands and receptors, it will be a challenge to develop experimental models which fully define the role of IGFBPs in development.

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